FEMALE PREFERENCE FOR ARTIFICIAL COURTSHIP SONGS IN 'DROSOPHILA MELANOGASTER' AND 'D. SIMULANS'

Elizabeth J. Halsey

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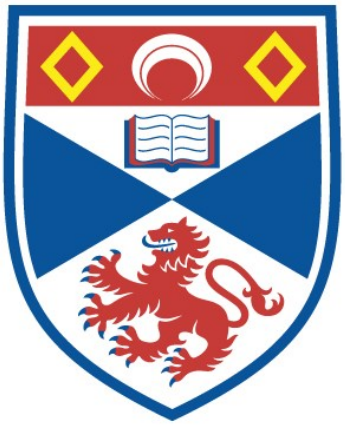

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Thesis submitted for the degree of Master of Philosophy

School of Environmental and Evoluntionary Biology
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Abstract

Fertilisation between species may be prevented by differences in their mating systems, if a mating signal of one species is not recognised as such by another species. Closely related species are often most differentiated in their mating systems. Speciation may come about as a consequence of divergence of mating signals and preferences of species. Reinforcement of differences in mating systems may be a consequence of sympatry of sibling species. Understanding mating systems, particularly in terms of how they contribute to reproductive isolation, is therefore central to the study of speciation and animal diversity.

Visual, chemical and auditory stimuli are required for mate-recognition in the Drosophila melanogaster complex. Among these stimuli is a species-specific male auditory signal, pulse song. Female preference for conspecific mean interpulse-interval (IPI) and IPI rhythm may contribute towards reproductive isolation of species, and divergence in this aspect of the mating system may be an important cause or effect of speciation of fruit flies.

The two chapters of this thesis examine different aspects of female preference for pulse song. The first chapter considers the contribution made by female preference for conspecific pulse song towards the sexual isolation of the sibling species *Drosophila melanogaster* and *D. simulans*. Synthetic pulse songs are shown to be most effective in stimulating females when they are of conspecific mean IPI and IPI cycle length. Preferences for conspecific signals are found to be broad. In the second chapter, it is asked which element of IPI periodicity accounts for its property as a sexual stimulant in *D. melanogaster*. The most likely explanation, increased variation in IPI, is eliminated: female receptivity is not increased by playback of a novel synthetic song which varies to the same degree as rhythmic song but does not oscillate. In an appendix, some differences between *D. sechellia* and *D. simulans* in microsatellite loci are reported.

Contents

General Introduction

For a group of animals to be described as a species, they must, by most definitions, show some degree of reproductive isolation (Templeton 1989). Mechanisms by which closely related species are isolated often occur prior to copulation, such that it is behavioural interactions that prevent production of hybrids. Pre-mating isolation may occur as a consequence of differences between species' mating signals and preferences, if individuals fail to be stimulated by heterospecific mating signals (Paterson 1985). Closely related groups of species are often most differentiated in their mating systems, to such an extent that their classification may be based most reliably on mating signals as opposed to traditional morphology (Ritchie and Gleason 1995). New species may arise when mating signals and/or preferences diverge. Alternatively, dysfunction of hybrids may bring about selection against behaviours permitting heterospecific copulation, and reinforce differences in mating systems of sympatric species. Species mating systems are therefore of central importance to studies of speciation and animal diversity, especially in terms of how they contribute to reproductive isolation.

Many attributes of the Drosophila melanogaster complex make it a good paradigm of pre-mating isolation. Precise study of behaviour is often made difficult by its only being amenable to qualitative assessment. However in these species, sexual behaviour is induced and quantified under experimental conditions with relative ease, reliability and little distortion between the field and the laboratory. In particular, chemical aphrodisiacs and auditory signals are easily scored, and courtship behaviour itself consists of discrete sub-units of behaviour suited to observation and measurement. Fertile interspecific hybrids can often be produced (Lemeunier *et al.* 1986) and a wealth of genetic markers and techniques are available for use, enabling the genetic basis of traits involved in sexual isolation to be elucidated (e.g. Kyriacou and Hall 1986; Liu *et al.* 1996; Coyne and Charlesworth 1997; Schug *et al.* 1998). Numerous mutants have been identified for pheromones (e.g. Cobb and Fervour 1996), courtship song (e.g. Kyriacou and Hall 1980; Villela and Hall 1996) and courtship behaviour (Hall 1994). Finally, two species of the complex are geographically isolated and two are often sympatric, enabling the importance of different selective forces shaping courtship traits to be assessed. These features of the melanogaster complex have led to its being extensively studied in the context of species isolation, providing a large amount of background information, including a detailed phylogeny of the complex.

A stereotyped sequence of interactions must take place before two individuals of the Drosophila melanogaster complex copulate. Visual, chemical and auditory stimuli are required. Each sex has a defined role in mate-recognition. Differences between species in some of these mating signals lead to sexual isolation. Among these mating stimuli is a male auditory signal, pulse song. The mean interpulse-interval (IPI) and IPI periodicity of pulse song differ between sibling species. Female preference for conspecific pulse song may prevent acceptance of heterospecific males as mates, and is therefore likely to contribute towards reproductive isolation. Speciation in fruit flies may often be a product of divergence in this aspect of the mate-recognition system. Divergence may take place in geographically isolated populations, or by reinforcement of species mate-recognition systems in sympatry.

Auditory signals have been investigated in a range of animal species by playback of signals. This technique allows responses to auditory signals to be observed while controlling for behaviours which would normally be associated with the emission of the signal. Stimuli can be recorded from real animals and played back. Alternatively, signals can be synthesised, in which case they can be either designed to imitate the original stimuli, or a novel signal can be concocted.

Female preference for pulse song is investigated here using playback of artificial 'songs'. The first chapter is concerned with the sexual isolation of the species of the melanogaster complex. It is asked whether female preference for conspecific pulse song contributes towards reproductive isolation of the sibling species *Drosophila melanogaster* and *D. simulans*. Playback of pulse songs synthesised to mimic the songs of the respective species is carried out, and the results discussed against an evolutionary background.

In the second chapter, closer attention is paid to female preference for rhythmic variation in IPI. Whether this feature of pulse song actually exists and could possibly play a role as a specific mating stimuli has been controversial. However, recent corroboration of the existence of pulse song oscillation has been published (Alt *et al.* 1998) and playback of artificial rhythmic pulse song is shown here to stimulate females. A novel pulse song is tested with a view to identifying the features of IPI periodicity responsible for its property of stimulating females.

Chapter One

The role of female preference for pulse song in the reproductive isolation of *Drosophila melanogaster* and *D. simulans*

(1.1.1) A REVIEW OF THE LITERATURE

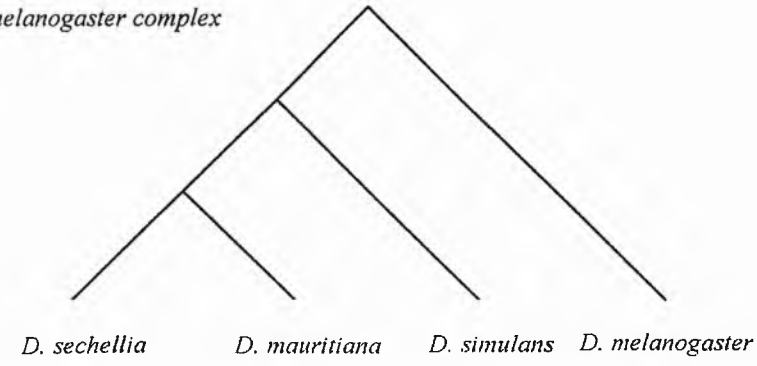
BACKGROUND: THE MELANOGASTER COMPLEX

Four closely related species comprise the melanogaster complex of *Drosophila*: *D. mauritiana, D. melanogaster, D. sechellia* and *D. simulans*. *D. melanogaster* is the most distinct of the four species. The phylogenetic relationships of the remaining three species are unclear: restriction mapping of mitochondrial DNA and sequencing of nuclear DNA indicate that the three pairwise genetic distances are nearly identical, such that it may never be possible to resolve beyond doubt the evolutionary history of the three species. However, biochemical, chromosomal and DNA hybridisation data indicate that *D. mauritiana* and *D. sechellia* are closer to one another than to *D. simulans* (Coyne and Kreitman 1986; Lemeunier *et al.* 1986; Caccone *et al.* 1996; figure 1.1.1). Given the distance separating the islands, perhaps the most likely scenario is two separate colonisations of the Seychelles and Mauritius by ancestral populations of *D. simulans* at approximately the same time. The four species of the melanogaster complex are very similar morphologically, and can be reliably distinguished only by shape of genitalia (Liu *et al.* 1996).

D. melanogaster and *D. simulans* are world-wide human commensal species, which originated in sub-Saharan Africa and spread with humans to Asia, Europe, America and Australasia. The two species are often sympatric in tropical regions (Lemeunier *et al.* 1986). The two other species are endemic to islands of the Indian Ocean: *D. mauritiana* to Mauritius and *D. sechellia* to three islands of the Seychelles. The two island species are believed to be allopatric to all other species of fruit fly (Lemeunier *et al.* 1986; Jallon and David 1987; Lachaise *et al.* 1988).

Feeding and sexual activity occur predominantly upon fermenting masses of fruits, to which fruit flies are opportunistically attracted by olfaction. Where more than one

FIGURE 1.1.1 *Phylogeny of the melanogaster complex*
(Not to scale)



species inhabit an area, they are found on the same food sites (Speith 1974; Ewing 1983; Lemeunier *et al.* 1986; Gromko and Markow 1993; Das *et al.* 1995). In addition to its geographical separation, *D. sechellia* is isolated from the other species by its exclusive preference for the fruits of the shrub *Morinda citrifolia* (Lachaise *et al.* 1986).

MATE-RECOGNITION AND PRE-MATING ISOLATION IN THE LABORATORY

Mate-recognition between two flies of the melanogaster complex is less likely to succeed if they are of different species; they are sexually isolated (e.g. Robertson 1983; Lachaise *et al.* 1986; Welbergen *et al.* 1992). This can be accounted for by neither temporal differences in sexual receptivity (see the next paragraph) nor failure of intromission, as interspecific hybrids are formed under particular conditions (Lemeunier *et al.* 1986). Therefore, the underlying cause of pre-mating isolation of species must lie in the series of interactions which always occurs between a male and a female before copulation (Sturtevant 1915). A male will seek out a female, investigate her briefly, then either ignore her or initiate 'courtship' behaviour. In response, the female will either allow copulation to occur or reject the male's advances. Therefore, both sexes can influence the outcome of an encounter, but primarily at different stages: the male, as the initiator of courtship, may fail to be induced to court a female at the outset, whereas it is the female who determines whether or not a courtship is consummated. There might, then, be seen to be two discrete levels, corresponding to roles of the two sexes, at which mate-recognition can fail between a pair of flies. The relative importance of each sex in pre-mating isolation will be examined separately.

SPECIES-SPECIFIC PATTERNS OF DIURNAL VARIATION IN SEXUAL RECEPTIVITY

Fixed temporal patterns of sexual activity can act as a means of species isolation in sympatric species (Butlin and Ritchie 1994). Species-specific restriction of mating to different times within the diurnal cycle might function as an isolating mechanism of *D. melanogaster* and *D. simulans* (Hardeland 1972). In the laboratory, females exhibit species-specific diurnal variation in sexual receptivity. For example, the sexual activity of *D. melanogaster* is greatest during the hours of darkness, whereas mating in *D. simulans* takes place almost exclusively during the day (Hardeland 1972). However, widening the perspective to include observations made in the field lessens the plausibility of this hypothesis. Most mating occurs on food sites, which

are visited diurnally for short periods only. During the night and remaining hours of the day, flies usually retire to secluded sites (Speith 1974). It would therefore be surprising if *D. melanogaster* copulate primarily at night under field conditions. In addition, those courtships of *D. melanogaster* that occur in the dark are less successful than those taking place in light (Burnet and Connolly 1974). Species-specific differences in sexual receptivity around the diurnal clock are unlikely to account for pre-mating isolation.

THE ROLE OF THE MALE IN SPECIES MATE-RECOGNITION: PHEROMONES

Male *Drosophila* tend to seek out females, or if two flies randomly encounter each other, it is the male which tends to initiate courtship. Any nearby individual resembling a fruit fly that is between half and double the male's own size will be investigated (Manning 1959a; Speith 1974); there does not appear to be any discrimination at the level of morphology, as is indicated by the lack of morphological differentiation between the sexes and species (Coyne and Kreitman 1987). The first action of a male is to tap the individual under investigation on its abdomen, thorax or head with his fore-tarsi. The fore tarsi of males bear chemoreceptors (Speith 1974). It is thought that tapping is a means by which males detect long-chain hydrocarbons which are found on the cuticle. These hydrocarbons have been found in species of the melanogaster complex to increase male propensity to court females; they play an aphrodisiac or pheromonal role (Jallon 1984). It would appear that hydrocarbons evolved to protect against desiccation (Coyne and Charlesworth 1997) and were subsequently adopted by males as means of recognising females. This secondary function of surface hydrocarbons seems to have occurred in many species of insect.

The predominant cuticular hydrocarbons of adult females differ between some species of the melanogaster subgroup (Jallon 1984; Jallon and David 1987; Cobb and Jallon 1990; table 1.1.1.1). Hydrocarbons tend not to differ within a species to the extent they differ between species, although there is some variation between geographic strains (Cobb and Jallon 1990). In some cases, male courtship is induced only by the pheromones of conspecific females and not those of heterospecific females (Jallon 1984). For example, under laboratory conditions, *D. simulans* males will court any female of the subgroup bearing 7-tricosene but none of the other species (Cobb and Jallon 1990; table 1.1.1.1). Males of some species, including *D.simulans,* can be induced to mate with females of a species from which they are normally isolated by the transfer of female pheromones from conspecific females (Coyne *et al.* 1994). The

species-specificity of female cuticular hydrocarbons might therefore contribute towards sexual isolation (Cobb and Jallon 1990).

TABLE 1.1.1.1 *Predominant female pheromones of species of the melanogaster subgroup*[*]

Species	Female hydrocarbon
D. mauritiana	7-tricosene
D. orena	7-tricosene
D. simulans	7-tricosene
D. teissieri	7-tricosene
D. yakuba	7-tricosene
D. melanogaster	7, 11-heptacosadiene
D. sechellia	7, 11-heptacosadiene
D. erecta	9,23-tritricontradiene

However, pheromonal cues cannot be responsible for sexual isolation between species in which female hydrocarbons do not differ. Males of *D. mauritiana, D. orena, D. simulans, D. teissieri* and *D. yakuba* will court females of any of these five species with equal vigour (Cobb and Jallon 1990; table 1.1.1.1). Only males of *D. erecta*, with its peculiar pheromone blend, are indifferent to all but conspecific females (Cobb and Jallon 1990). Moreover, stimulation by hydrocarbons is non-specific in some cases: *D. melanogaster* and *D. sechellia* males court females of all species in the subgroup (Welbergen *et al.* 1992; Butlin and Ritchie 1994; Coyne and Charlesworth 1997). Furthermore, data described below will indicate that the courtship of *D. simulans* males is less discriminate than the literature reviewed above supposes. Therefore, assuming that any discrimination made by males occurs prior to courtship, sexual isolation of these species occurs as a consequence of the female declining to accept the male as a mate.

THE ROLE OF THE FEMALE IN SPECIES MATE-RECOGNITION: COURTSHIP

Upon recognising a female as a potential mating partner, a male will direct various stimuli at her. This stereotyped courtship behaviour can be decomposed into sub-units, which differ quantitatively and qualitatively between species. The following courtship routine is characteristic of the melanogaster subgroup. First, a male will position himself near to the female such that his body axis is directly toward her, and will follow her should she move. He may then move in a small arc around the female, performing other courtship behaviours while doing so. The wings play an important part on courtship: by contraction of the flight muscles in the thorax, a male will extend and vibrate one or both wings up and down for brief periods. This action directs a sound toward females termed 'courtship song'. A male may also 'scissor'

[*] Jallon 1984; Jallon and David 1987; Cobb and Jallon 1990

his wings by rapidly opening and closing them for a few seconds. Finally, towards the end of courtship, brief contact is often made between the male's proboscis and the genitalia of the female (Cowling and Burnet 1981; Cobb *et al.* 1989).

After licking a female, the male may attempt to copulate. The success of this attempt depends on the behaviour of the female: she can accept, reject or prolong courtship by behaving ambivalently. Receptive females appear passive. Unreceptive females exhibit a number of rejection responses – within the melanogaster subgroup the following are observed: wing flicking or separating, leg-kicking, fending, abdomen-raising, vaginal plate-extrusion and decamping (Speith 1974; Cobb *et al.*, 1989). These are less likely to be signals than physical barriers to copulation in *D. melanogaster* (Ewing 1983). The few males that attempt to mount a female displaying rejection responses are rarely successful (Bastock and Manning 1955; Speith 1974; Gromko and Markow 1993). In addition, an acoustic signal, 'rejection song', which causes males to turn away from females, is found among the repertoire of female rejection signals (Ewing and Bennet-Clark 1968).

Courtship stimuli have been identified by measuring the sexual competency of various mutant or surgically modified individuals against normal controls. When both the aristae, which are required for auditory perception (Manning 1967b), of females are removed, mating is drastically suppressed in *D. melanogaster* (Mayr 1950; Manning 1967b; Burnet *et al.* 1971; Markow 1987) and *D. sechellia* (Cobb *et al.* 1989). Similarly, removal of the wings of males, rendering them mute (Schilcher 1976a), results in a great reduction in mating (Sturtevant 1915; Shorey 1962; Manning 1967b; Kyriacou and Hall 1982; Cobb *et al.*, 1989). Males of vestigial wing phenotypes also have low mating success (Ewing 1983). Wingless and intact males fare equally well when their mating competency is tested using aristaless females (Bastock 1956). Mate-recognition is therefore less efficient when a means of either signalling or receiving an auditory mating signal is removed. A clear candidate for such an auditory stimulus is courtship song.

COURTSHIP SONG

The courtship songs of species of the melanogaster subgroup have two major components: a humming sound, rather like the sound made by flight but of a lower frequency, and a succession of pulses. The former is known as 'hum song' or 'sine song' and the latter is referred to as 'pulse song' or 'love song' (figure 1.1.1). Pulse song is produced by only one wing whereas sine song, which is of higher amplitude,

is produced by both wings. The courtship songs of *D. sechellia* and *D. yakuba* are peculiar among the species of the subgroup in not containing sine song (Cobb *et al.* 1989). Courtship song is found across a wide range of *Drosophila* species (Ewing 1983).

The low mating success of wingless males can be partially restored by playing artificial pulse song to females as they are courted (Bennet-Clark and Ewing 1967, 1969; Burnet *et al.* 1971; Schilcher 1976b; Kyriacou and Hall 1982, 1984, 1986; Greenacre 1993). This effect is not seen when random, 'white' noise is played to females (Kyriacou and Hall 1982). Artificial sine song increases sexual receptivity, but only when it is played prior to courtship (Schilcher 1976b; Kyriacou and Hall 1984). Both pulse song and sine song would appear to be components of the mate-recognition system. Because reproductive fitness is highly dependent upon the ability to recognise members of the opposite sex, traits influencing the efficiency of mating are likely to be under strong selection and are therefore predicted by have low genetic variability (Fisher 1958). The low phenotypic and genetic variability of IPI within populations of *D. melanogaster* therefore substantiates its proposed role as a mating signal (Ritchie and Kyriacou 1994).

The time interval between pulses (figure 1.1.2), usually reported in terms of 'interpulse-interval' (IPI), varies methodically (Shorey 1962). Pulses occur more frequently for a time before reaching a peak then gradually becoming less frequent, such that a sinusoid can be fitted to estimates of mean IPI (Kyriacou and Hall 1980; Alt *et al.* 1998). There is variation between species in mean IPI (Ewing and Bennet-Clark 1968; Cowling and Burnet 1981) and IPI length cycle (Kyriacou and Hall 1980) (table 1.1.1.2). This species-specificity of mean IPI extends across many species groups of *Drosophila* (e.g. Waldron 1964; Miller *et al.* 1975; Ewing 1983; Ewing and Miyan 1986; Tomaru and Oguma 1994; Ritchie and Gleason 1995).

TABLE 1.1.1.2 *Estimated mean IPIs and IPI cycle lengths of species of the melanogaster complex*

Species	Mean IPI (ms)	Mean cycle length (s)
D. melanogaster[1]	30-34	55
D. sechellia[2]	70-90	70
D. simulans[3]	48-57	35

[1] Ewing and Bennet-Clark 1968; Cowling and Burnet 1981; Kyriacou and Hall 1982, 1986
[2] Cobb *et al.* 1989; C. P. Kyriacou, pers. comm.
[3] Ewing and Bennet-Clark 1968; Cowling and Burnet 1981; Kyriacou and Hall 1982, 1986

That song characteristics tend to be species-specific makes them likely species-recognition signals (Waldron 1964). Accordingly, the efficiency of pulse song as a mate-recognition signal is dependent on the length of its mean IPI and IPI periodicity in some species; the capacity of artificial pulse song to supplement the courtship of wingless males is maximum when its IPI is of the conspecific length. This has been found in both *D. melanogaster* (Bennet-Clark and Ewing 1969; Schilcher 1976b; Kyriacou and Hall 1982) and *D. simulans* (Kyriacou and Hall 1982), in addition to species outside of the melanogaster complex, such as *D. biauraria* (Tomaru *et al.* 1995) and *D. parabi-pectinata* (Crossley and Bennet-Clark 1993).

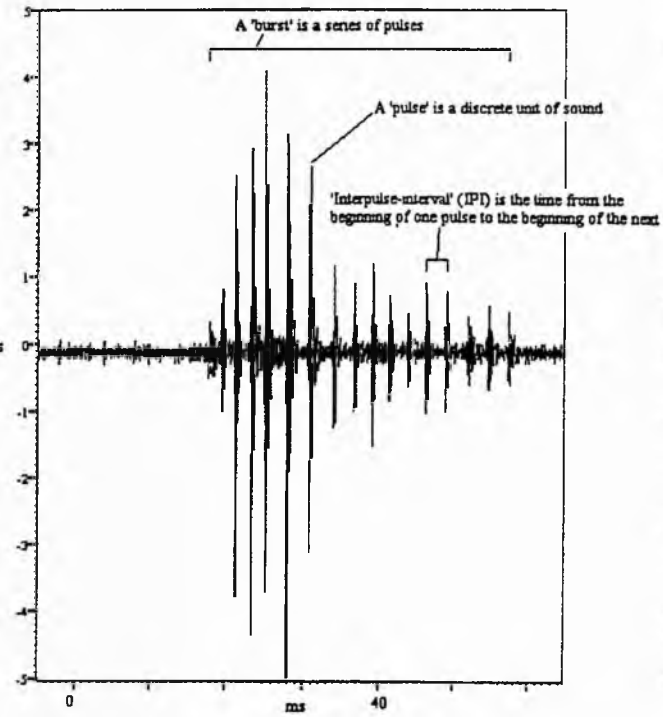


FIGURE 1.1.2 Pulse song typical of *D. melanogaster.*

Songs that incorporate cycling of IPI increase female receptivity most if cycles are of conspecific cycle length in D. melanogaster and D. simulans (Kyriacou and Hall 1982, 1986; Greenacre et al. 1993). The influence of IPI cycle length on female receptivity will be considered more fully in chapter 2. Sine song, which does not differ between species, is unlikely to play a role in pre-mating isolation (Kyriacou and Hall 1982; Cobb et al. 1989).

It might therefore be supposed that when a male courts a female, she is more likely to recognise him as a mate if he emits pulse song. This signal is more efficient at particular IPI specifications , which differ between species. When a female is courted by a heterospecific male, the pulse song she encounters is more likely to deviate from the required specifications. As a consequence, the mate-recognition system is more likely to fail, the female more likely to commence rejection behaviours and copulation less likely to occur. In short, the species-specificity of IPI and preference for IPI contribute to reproductive isolation.

EXTRAPOLATION OF LABORATORY OBSERVATIONS TO THE FIELD

The species-specificity of two elements of the mating system, female pheromones and male pulse song, may be primarily responsible for the failure of heterospecific individuals to recognise one another as mates in the laboratory. These behaviours will

individuals to recognise one another as mates in the laboratory. These behaviours will now be placed in a wider context. Effects of laboratory conditions and relevant observations made in the field will be reviewed. It will be argued that this wider setting support the proposed role for courtship song as a species mate-recognition signal.

Accounts of sexual behaviour given this far have been based on observations made under 'no-choice' conditions: two individuals, one of each sex, are placed together and their subsequent behaviour is observed. Under these circumstances, as was noted in earlier, males of *D. melanogaster* are seen to court females of both species, whereas *D. simulans* males court only conspecific females (e.g. Manning 1957a; Barker 1967; Wood and Ringo 1980; Welbergen *et al.* 1992; Butlin and Ritchie 1994). However, when a male-biased sex-ratio or a mass-mating technique is employed to assess the direction of pre-mating isolation, the direction of the asymmetry apparently reverses: female hybrids are produced in greater numbers (e.g. Sturtevant 1929; Manning 1959a; Lemeunier *et al.* 1986; Das *et al.* 1995). As the sex of hybrids is usually that of the *D. melanogaster* parent (Sturtevant 1920; Lemeunier *et al.* 1986) this indicates that the cross between *D. melanogaster* females and *D. simulans* males occurs more commonly than its reciprocal. This apparent contradiction is possibly resolved by paying close attention to the ages of the males and females. Barker (1967) found that *D. simulans* males courted *D. melanogaster* females when they are one day old but not when five days old. Furthermore, hybrids were produced at similar frequencies in crosses of both directions when ages were standardised except when one day-old females and five day-old males were used. In this latter case, the cross between *D. simulans* females and *D. melanogaster* males produced more hybrids than the reciprocal cross. Therefore, the reputed differences in frequency of hybridisation may result from species differences in male courtship at different ages, perhaps because the relevant cuticular hydrocarbons of very young *D. melanogaster* females are not present in sufficient quantities to deter *D. simulans* males from courtship. The level of discrimination by females may differ between the two species. Certainly, female behaviour differs generally between single-pair and crowded conditions (e.g. Speith 1974)

Considering the extent to which the mating behaviour of *Drosophila* has been studied in the laboratory, a very small amount of data are available on sexual activity in the field. There are, however, some records that bear relevance to the present study. First, females are liable to find themselves courted by males of other species. Gromko and Markow (1993) observed courtship in a sympatric population of *D. melanogaster* and *D. simulans*. The frequency of heterospecific courtship was that expected if

courtship is initiated independently of species; males did not appear to be exercising any discrimination. This would be expected of *D. melanogaster* but not *D. simulans* males, if it is pheromonal cues that determine whether a male is stimulated. Furthermore, young, sexually immature males of the two species court both other males and conspecific females in the laboratory, as the specificity of male courtship is experience-dependent (Speith 1974; McRobert and Tompkins 1988; Vaias *et al.* 1993). Male courtship may therefore be less specific than is often assumed on the basis of laboratory observations. Second, only a small proportion of courtships end in copulation (Gromko and Markow 1993). Third, the great majority courtships are terminated by female-initiated and not male-initiated behaviour (Ewing 1983; Gromko and Markow 1993). Finally, the frequency of hybrids found in the field is very much lower than that expected under random mating (Speith 1974; Lemeunier 1986; Welbergen *et al.* 1992; Das *et al.* 1995). Although this may reflect lower fitness of hybrids, it is possible that a contributory factor is a deficiency of heterospecific copulations relative to homospecific copulations.

It might, on the basis of these observations, be contended that females in sympatric populations are frequently courted by males of other species, that homospecific copulation is more frequent than heterospecific copulation, and that female rejection of males is the primary means of determining which courtships are consummated in the field. Therefore, females may favour the courtship of conspecifics over males of other species in the field.

Moreover, there is evidence that differences in mean IPI result in sexual isolation in the field. Individuals taken from allopatric populations of *D. melanogaster* mate randomly with each other (Henderson and Lambert 1982) and mean IPI does not differ to any great extent between populations (Ritchie *et al.*, 1994). However, a population sexually isolated from other strains was discovered in Zimbabwe (Wu *et al.* 1995), was found to have a much lower mean IPI than is typical (Pugh 1997). In view of the playback experiments reported above demonstrating female preference for species-specific artificial pulse song and apparently indiscriminate courtship of *D. melanogaster* males, this constitutes strong evidence that differences in mean IPI and preferences for IPI are important in sexual isolation.

THE EVOLUTION OF COURTSHIP SONG DIFFERENCES

Differences in IPI and preference for IPI have been argued to contribute towards reproductive isolation of species in the laboratory and the natural environment.

Changes in IPI and preferences for IPI may therefore lead to the formation of new species. What causes mating systems to diverge? The species of the melanogaster complex will be used to illustrate four major evolutionary mechanisms of divergence.

CHANGES OF MATING SYSTEMS IN ALLOPATRY

Given that *D. sechellia* is geographically and ecologically isolated from all other species of fruit fly, interactions with other species are unlikely to have played a major role in the evolution of its courtship traits. Yet, at more than 70 milliseconds, its mean IPI is more removed from those seen in closely related species (table 1.1.1.2). While assumptions about evolutionary history should not be made freely, this would seem to infer that intraspecific interaction alone could result in changes of IPI. What kind of forces might act on a mate-recognition system in allopatry? A first possibility is that ecological factors play a role in shaping courtship song. Environmental selection is perhaps less likely to influence pulse song directly than, say, the visual male secondary sex traits in guppies (e.g. Endler and Houde 1995). However, a mate-recognition system signal should not be thought of as being isolated from other aspects of the organism. For example, pulse song is produced by the muscles responsible for flight (Bennet-Clark and Ewing 1968), and signal receptors typically have functions in addition to the reception of mating signals (Butlin and Ritchie 1994). Traits involved in mate-recognition may therefore be influenced indirectly by ecological selection. Extending this line of thinking to a developmental or genetic level, mate-recognition systems might be influenced by pleiotropic effects of genes controlling phenotypically unrelated traits sensitive to external selection. For example, mutation of the *period* gene of *D. melanogaster* can affect both IPI cycle length and length of diurnal cycle (Kyriacou and Hall 1980). Alteration of one trait of an organism does not necessarily take place independently of other, manifestly unrelated characteristics of an organism (Bonner 1982). However, the phenotypic constancy of mean IPI across allopatric populations of *D. melanogaster* (Ritchie *et al.* 1994), and *D. teissieri* (Paillette *et al.* 1997) may be taken to indicate that pulse song is relatively unsusceptible to ecological influences.

A second force likely to contribute towards divergence of mate-recognition systems is sexual selection (Andersson 1994). Sexual traits are often found to differ more between closely related species than other characteristics (Butlin and Ritchie 1994), as is the case with IPI in *Drosophila*. Sexual traits and preferences are expected to change continually as a result of Fisher's runaway process (Fisher 1958; Lande 1982). Mate-recognition systems are predicted to diverge in allopatric populations

independently of ecological differences and genetic drift (Iwasa and Pomiankowski 1995). Because the process has no inherent bias, the mating systems of allopatric populations are likely to diverge. Sexual selection seems to have contributed towards diversity of mating systems in Hawaiian species of *Drosophila* (Butlin and Ritchie 1994) and to song in the *D. willistoni* species group (Ritchie and Gleason 1995). However, the runaway process requires there to be genetic coupling of traits and preferences (Lande 1982), for which there is little empirical evidence (Butlin and Ritchie 1994). Nevertheless, sexual selection is probably a major contributor to the considerable diversity of IPI signals in many closely related groups of fruit fly.

Thirdly, there are processes to which a newly established or bottlenecked population is subject on the basis of its small size, such as genetic drift and founder effects. A small, 'sample' population is likely to contain only a subset of the genetic variation present in the parental population, leading to an initial bias ushering a trait down a particular evolutionary path. In addition, other peculiarities of an expanding population in a new environment may increase the likelihood of diversification in mating systems (Carson and Templeton 1984).

All three of the evolutionary mechanisms of divergence described above could have influenced the mating system of *D. sechellia*. A new microhabitat has been adopted by the species, possibly reflecting or introducing ecological selection on the system directly or indirectly. Sexual selection is also a possible influence; coupling of mating traits and preferences is perhaps inferred by the unusually strong discrimination exercised by females against mating with males from other species (Lachaise *et al.* 1986), although it is not known whether this is a function of the IPI differences between species. The founding population(s) colonising the islands of the Seychelles may have been small, and therefore subject to founder effects and genetic drift. It may be of relevance here that *D. sechellia* probably descended directly from *D. simulans*, which is relatively divergent for IPI (Kawanishi and Watanabe 1980; Kyriacou and Hall 1986); a 'sample' *D. simulans* population is more likely than, say, *D. melanogaster* to contain an atypical collection of 'song genes' from the original population. The mean IPI of the closely related *D. mauritiana*, at approximately 46 milliseconds (Cowling and Burnet 1981), has not evolved in the same direction as *D. sechellia*. Divergent mean IPI has been found in isolated populations of *D. melanogaster* (Ritchie *et al.* 1994; Pugh 1997), which may be on their way to becoming new species.

SELECTION FOR DIVERGENCE OF MATING SYSTEMS IN SYMPATRY

Unlike *D. sechellia*, *D. melanogaster* and *D. simulans* often coexist in sympatry and there are no ecological barriers between the species. In these populations there will be frequent encounters between flies of different species, presenting opportunities for heterospecific copulations to occur. Hybrids between these species are sterile (Sturtevant 1920; Lemeunier *et al.* 1986), so behaviour permitting copulation with a fly of another species is strongly maladaptive. As a consequence of differences in the cost of reproduction between the sexes (Bateman 1948), this is particularly true for females. Natural selection is expected to modify mate recognition signals to favour recognition of conspecifics over heterospecifics as mates; an increase in pre-mating isolation is expected (Dobzansky 1937). This is in contrast to allopatric models, in which sexual isolation is an incidental consequence of divergence in mating-recognition systems. There is circumstantial evidence that selection resulting from hybrid dysfunction in *Drosophila* has increased pre-mating isolation in sympatry (Coyne and Orr 1989; Noor 1995), led to pheromonal divergence (Jallon and David 1987) and narrowed the range of IPIs which function as a mate-recognition signal in *D. biauraria* (Tomaru *et al.* 1995). The courtship displays of *D. melanogaster* and *D. simulans* are less variable than the allopatric species of the melanogaster complex (Cobb *et al.* 1989), perhaps reflecting selection for greater specificity of courtship where the possibility of mis-mating exists (Mayr 1970).

However, pre-mating isolation of species does not necessitate an explanation of reinforcement or reproductive character displacement. Allopatric species such as *D. mauritiana* and *D. sechellia* can be sexually isolated from other species of the melanogaster complex (Robertson 1983; Lachaise *et al.* 1986; Coyne and Charlesworth 1997), and geographic clines of 'crossability' of *D. melanogaster* with *D. simulans* are found where *D. simulans* does not exist (Das *et al.* 1995). Intraspecific processes are most likely to account for differences in mate-recognition systems in these cases, which are unlikely to be exceptional. That the genetic basis of the difference between *D. mauritiana* and *D. simulans* in IPI is polygenic, as opposed to being based on a few 'major' genes, infers that IPI diverged gradually rather than abruptly (Orr and Coyne 1992; Pugh and Ritchie 1996), as is perhaps more fitting with models of allopatric divergence than models of reinforcement.

Four evolutionary processes by which species mate-recognition systems might diverge have been described: ecological selection, sexual selection, processes acting

on small populations and selection against hybridisation. Pre-mating isolation - that is, failure of flies to recognise one another as mates - in the laboratory between geographically isolated species is probably a consequence of allopatric divergence, whereas the mate-recognition systems of sympatric species may be more divergent as a consequence of selection against hybridisation. The relative importance of each force is difficult to ascertain, and likely to vary between systems.

SUMMARY AND CRITICISMS

The sibling species of the melanogaster complex are sexually isolated under laboratory conditions. Female preference for conspecific pulse song may account for this, as IPI and IPI cycle length are species-specific, females do not mate indiscriminately with respect to species, and playback most effectively increases female receptivity when pulse song is of conspecific mean IPI and IPI cycle length. Field observations and the effects of sex ratio and age of flies complicate matters, but arguably support a role for female preference in pre-mating isolation.

Species-specificity of a mating signal does not necessarily infer its importance in bringing about or maintaining sexual isolation. Noor and Aquadro (1998) suggest that species-differences in mean IPI arose primarily as pleiotropic side effects of adaptation to different ecological circumstances or intra-specific sexual selection, and play no role in species-isolation. These authors point out that IPI is easily influenced by small environmental changes, which would not be expected of a species-recognition signal. In addition, it is argued that genetic and phenotypic variability within and between populations is masked by individual variation in IPI, and is not as low as has been claimed previously. Their own observations of *D. persimilis* and *D. pseudoobscura*, which differ in mean IPI, indicate that mean IPI is not a criterion of female preference, although females discriminate between species. Furthermore, where sexual isolation of melanogaster subgroup species is concerned, recent emphasis has been on the role of male discrimination on the basis of female pheromones (e.g. Jallon 1984; Coyne *et al.* 1994; Coyne and Charlesworth 1997).

Differences in pulse song between species constitutes circumstantial evidence that it contributes to sexual isolation, which must be verified by direct tests of female preference for conspecific pulse song. Following sections will present experimental evidence that *D. melanogaster* and *D. simulans* females prefer conspecific mean IPI and IPI cycle length, and it will be argued that sexual isolation of species of the melanogaster complex is at least partly a consequence of this.

(1.1.2) EXPERIMENTAL AIMS AND RATIONALE

Female preference for conspecific pulse song may result in the sexual isolation of *Drosophila melanogaster* and *D. simulans.* This hypothesis was tested by measuring female sexual receptivity resulting from exposure to pulse songs of different species. Specifically, the following questions were asked:

- Is female sexual receptivity affected by pulse song?
- Does females sexual receptivity differ between pulse songs differing in IPI and IPI cycle length of magnitudes seen between the species of the melanogaster complex?
- Does the species of a female determine her sexual receptivity to different songs, such that conspecific song is the most effective?
- Do 'heterospecific' songs of sympatric and allopatric species elicit different responses?

Three effects of three 'songs', characteristic of three species of the melanogaster complex, *D. melanogaster, D. sechellia* and *D. simulans,* were compared against each other and a negative control of silence. Two of these species, *D. melanogaster* and *D. simulans,* are sometimes sympatric, and one, *D. sechellia* is allopatric. Ideally, all three species would have been tested. However, *D. sechellia* is not amenable to assays of mating preference, as it exhibits a very low rate of copulation under laboratory conditions (personal observation; Cobb *et al.* 1989). Therefore, only the preferences of *D. simulans* and *D. melanogaster* females were examined.

The effectiveness of different auditory signals in stimulating females has been measured by various means. For example, male mating success can be correlated with differences in song traits. This has been carried out using interspecific hybrid males (e.g. Noor and Aquadro 1998), mutant males (e.g. Barnes *et al.* 1998) and in males selected for song traits (e.g. McDonald and Crossley 1982), for example. However, correlation between song traits and other male traits associated with mating success may render findings inconclusive. Measuring the response of females to artificial courtship song can circumvent this problem. In some species of *Drosophila,* female sexual receptivity can be measured directly by acceptance postures (Speith 1974; Ritchie *et al.* 1998). However, the difference in behaviour of unreceptive and receptive females of the melanogaster complex is effectively undetectable (Bastock and Manning 1955), such that female sexual receptivity can be measured only in terms of rate of copulation. Females receptivity was measured here as rate of

copulation, or mating speed, with conspecific males incapable of making song themselves; that is, males which have had their wings surgically removed (Schilcher 1976a).

However, this protocol introduces the potentially confounding factor of male stimulation. Male *D. melanogaster* activity and courtship increase upon perception of simulated pulse song (Schilcher 1976a; Kyriacou and Hall 1982; Crossley *et al.* 1995). As a consequence of this, some experimenters have used males that have been 'deafened' by removal of their aristae (Schilcher 1976a), although there is also some doubt as to whether this altogether removes sensitivity to song, since aristaless males seem to perceive pulse song of amplitude greater than a level between 60 and 106 dB (Schilcher 1976a). Either way, differences in IPI do not influence the degree to which males are stimulated (Schilcher 1976a; Kyriacou and Hall 1982); the effect is non-specific. Therefore, *relative* differences in mating activity between songs relative to one another will not result from this phenomenon, unless there is interaction between male courtship intensity and female preference, and this was not found to be the case with *D. montana* (Ritchie *et al.* 1998). On these grounds, the aristae of males were not removed. Any differences in response to silence and pulse song will be magnified by the use of intact rather than 'deaf' males (Kyriacou and Hall 1982). Aside from the removal of stimuli produced by the wings, wingless males seem to court females normally (Kyriacou and Hall 1982).

Virgin females were used to ensure reliable and vigorous courtship by males (Bastock and Manning 1955). Females were tested in groups of twenty to maximise sample size.

Measurement of behaviour under laboratory conditions must be undertaken with care. The courtship behaviour of *Drosophila* is influenced by many factors, including age, temperature, humidity, time of day (e.g. Shorey 1962; Barker 1967; Cobb *et al.* 1987; Ritchie and Kyriacou 1994; Noor 1997), experimental apparatus (Robertson 1982; Ewing 1983) and method of confinement (Schilcher 1976a; Ewing 1983). The experimental conditions used here were far removed from circumstances in which mating behaviours would usually take place. Even so, previous laboratory observations of the mating behaviour of these species often agree closely with data collected in the field (Gromko and Markow 1993). Nevertheless, precautions were taken with a view to minimising variation in mating behaviour resulting from the environmental, including the use of flies with no prior sexual experience (Scott 1986; Noor 1997), standardised density in culture and confinement (Schilcher 1976a; Ewing 1983; Noor 1997), aspiration rather than carbon dioxide to transfer flies to apparatus,

divided chambers (Robertson 1982), a period of time allowing reduction of agitation (Robertson 1982) and large sample size. All four treatments were assayed within single sessions, and factors such as age and time of day were tested for effects on mating speed.

(1.2) MATERIALS AND METHODS

STOCKS

D. melanogaster and *D. simulans* adults were collected from long-term laboratory cultures which had been maintained on standard medium in 12:12 cycles of light and darkness. Stocks were kept at 25°C for the period of experimentation. Adults used for experimentation were collected within two hours of eclosion with the minimal use of carbon dioxide as anaesthesia, divided according to sex and aged in vials. The virginity of females was assured by rejecting any vial, which showed evidence of fertile eggs a few days into storage. Flies were aged for two to twelve days prior to their use (see below). No fly was anaesthetised less than twenty-four hours before use. Each fly was used for experimentation once only.

SYNTHESIS OF SONG

'Songs' were created using the *SIGNAL3* system (Electronic Design). All parameters except IPI, IPI cycle length and the amplitude of the song rhythm were kept constant between songs (table 1.2.1). All songs were constructed using the same pulse shape. IPIs and IPI cycle lengths of the three synthetic songs, corresponding to the estimates given earlier, are presented in table 1.2.2. The amplitude of each cycle was of magnitude +/-10% of the mean IPI of a song, as is approximately typical of real songs (Kyriacou and Hall 1980). Random 'noise' was added to each song to mimic the variation of IPI in real songs (e.g. Ewing 1983). As IPI was song-specific, and burst-length and pulse-length were constant, the duration of silence between bursts differed between songs, such that the interval between bursts was longest in 'melanogaster' song and shortest in 'sechellia' song. The three songs are depicted in figure 1.2.1 (overleaf)

TABLE 1.2.1 *Some parameters of synthesised songs*

Parameter	Value
Carrier-frequency	380 Hz
Pulse-length	10 ms
Inter-burst-interval	6 s
Number of pulses per burst	70
Pulse-decay	0.8 ms
Sampling-frequency	6000 Volts
Peak amplitude	3000 Volts

FIGURE 1.2.1 *The three artificial songs*

(a) 'melanogaster'; (b) 'sechellia'; (c) 'simulans'

Note: the vertical axes designate Hertz and not milliseconds

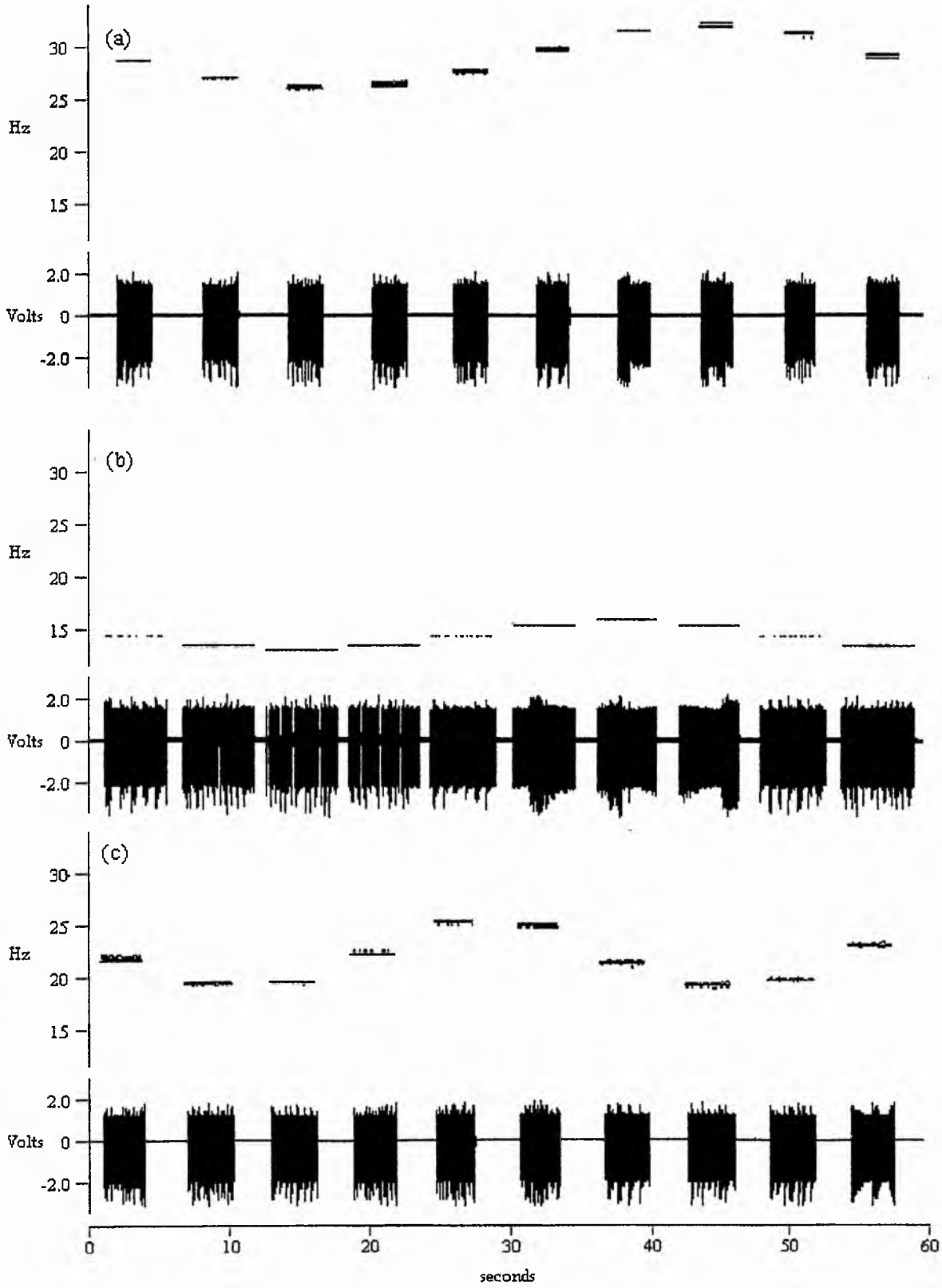


TABLE 1.2.2 *IPIs and IPI cycle lengths of synthesised songs*

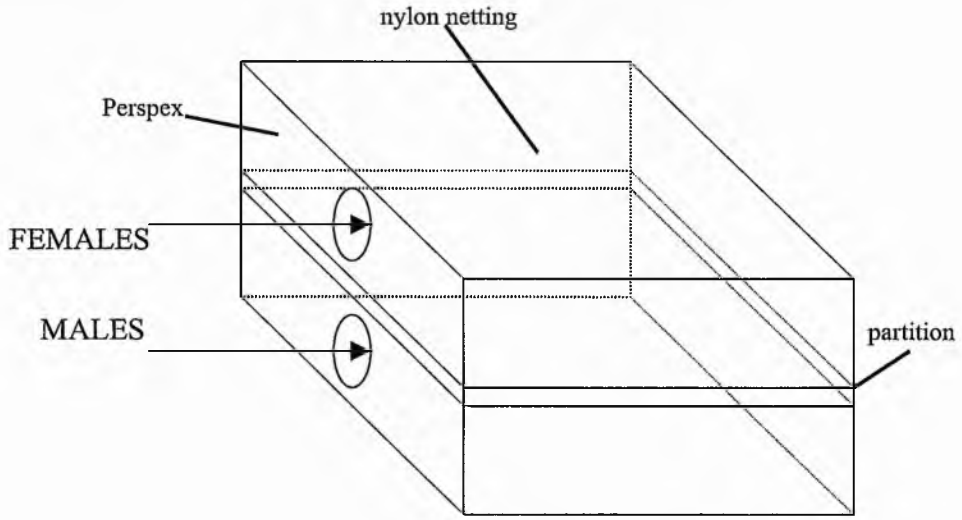
Song	Mean IPI (ms)	Mean IPI cycle length (s)
'melanogaster'	34	55
'sechellia'	70	48
'simulans'	48	35

Songs were filtered between 50 and 200 Hz, recorded onto Zonal 676 magnetic tape and amplified using a Tascam 22-2 reel-to-reel tape recorder. Four loudspeakers were tested for quality of amplification. The Boston Acoustics *MicroMedia* System was chosen as it produced the lowest level of background noise and preserved pulse shape most accurately.

EXPERIMENTAL PROTOCOL

Twenty females were aspirated into the upper section of a 'mating chamber' of inner dimensions 50 by 45 by 30 mm. The walls of the chamber consisted of transparent Perspex while the floor and ceiling were made from fine nylon netting through which sound could pass with little distortion. Twenty conspecific males were placed in the lower portion of the chamber, such that the flies were kept separate according to sex (figure 1.2.2). The wings of males had been removed one day prior to the trial. The chamber was suspended over the loudspeaker and the sound pressure level within the chamber, as monitored using a *Realistic Sound Level Meter 33-2050*, was kept constant around 70dB. This value was chosen because it elicited the highest rate of mating activity and because it is likely to approximate to the amplitude reaching females when being courted by males (Schilcher 1976a). The height of the chamber above the speaker was approximately 25cm.

FIGURE 1.2.2 *The mating chamber*



Following a period of three minutes intended to permit the flies to accustom themselves to their surroundings, a partition between the two groups was removed carefully and the flies became free to mix, at which time song playback was begun. The number of pairs of flies copulating was recorded at intervals of two minutes for a total period of twenty minutes. This length of time was chosen on the grounds that it is the approximate duration of copulation, and, as has been seen previously, is an appropriate time-span to observe this behaviour (e.g. Manning 1967b). Males do not normally dismount within this period of time. Temperature was maintained carefully at 25°C throughout each experimental period, as female preference for IPI is influenced by temperature (M. Ritchie, pers. comm.).

After each day of experimentation, chambers were cleaned with hot, soapy water to remove any residues of pheromonal or anti-aphrodisiac compounds. If a mating chamber were used more than once in a day, a stream of air would be passed through each chamber for a minute between trials. All the four synthesised songs to be tested within a given experiment would be played back within a period of two hours, and this constitutes one 'trial'. Each of the two experiments consisted of twenty trials, such that the sample size was 400 flies of each sex per song per species. Behaviour often fluctuates greatly in assays of this type and in view of this a large sample was necessary.

SECONDARY VARIABLES

Five 'secondary' variables were recorded for each trial: block, time of day, order, female age and male age:

Block Each of the three trials was carried out over a period of four to six weeks. Within each respective experimental period, there were two, three or four 'blocks'. Each block, designates a generation of flies which hatched within a few days of each other.

Time The time of day was given as one of three categories: '0' designated trials begun before 1100 hours, '1' designated trials begun before 1400 hours and '2' designated trials begun after 1400 hours. No trial was begun before 0900 hours or after 1600 hours.

Order The order in which the four songs constituting any one trial was decided using a table of randomly generated integers: 1 (the first song to be played back) to 4.

Age The age profile of the flies of the respective sexes was kept constant within trials. The difference between the maximum and minimum ages of flies used within a trial was reasonably constant between trials. The ages used in analysis are means.

The courtship latency and duration of *D. simulans* is lower than that for *D. melanogaster* (Manning 1959b; Cobb *et al.* 1987). Both latency and duration tend to decline as flies become older (Barker 1967; Cobb *et al.* 1987). The age of *D. simulans* flies required to observe difference between songs (indeed, required to observe any matings at all) was greater than that required of *D. melanogaster*. The mean ages of the flies of the respective sexes and species are given in table 1.2.3. The use of flies of these ages will be seen to standardise the rate of mating in the control condition of silence.

TABLE 1.2.3 *Mean age of flies (days)*

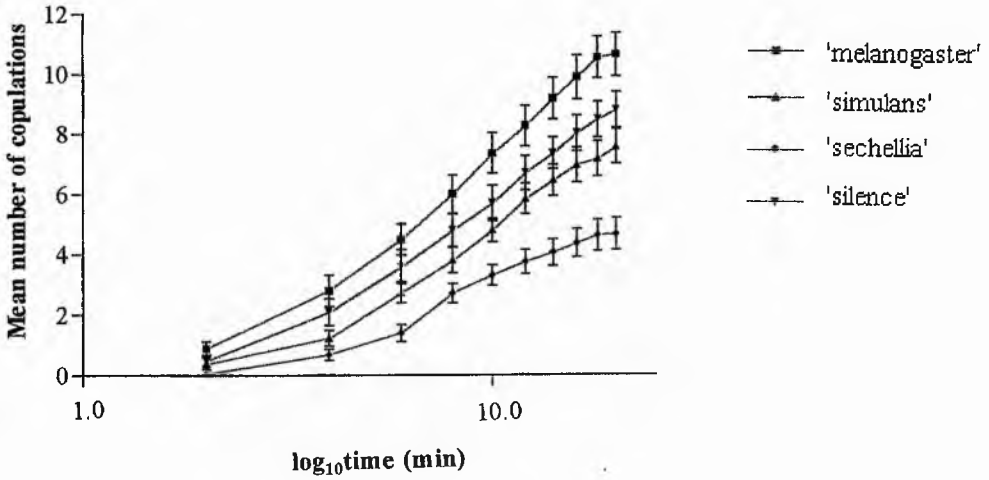
	Female	**Male**
D. melanogaster	4.3	4.2
D. simulans	8.3	8.4

STATISTICAL ANALYSIS

The bulk of the analysis was carried out using general linear modelling (GLM). As a linear relationship between variables is required, it was necessary to transform the data such that the mean number of copulations increased linearly with time. The data were most linear after logarithmic (base 10) transformation of time. Figure 1.2.3 shows a typical relationship between number of matings and the transformed time variable. For all data, the distribution of differences between the observed values and the fitted regression line was not significantly differently from values expected under a normal distribution (Kolmogorov-Smirnov tests for normality, $p<0.01$). The data therefore satisfy the assumptions of linear regression.

FIGURE 1.2.3 D. melanogaster *data after logarithmic transformation*

Bars represent standard errors



Although the data were orthogonal, GLM was used rather than ANCOVA because the former makes possible the testing of interaction of factors; specifically, GLM permits the incorporation of the 'SONG* LOG_{10}TIME'. A significant interaction term is, among other things, indicative of regression lines that intersect as opposed to run parallel to one another. If a model lacked interaction terms, as it would if ANCOVA were used, this distinction could not be made.

The GLM used here is:

NO. COPULATIONS = SONG LOG_{10}TIME SONG* LOG_{10}TIME BLOCK 'TIME OF DAY' ORDER 'FEMALE AGE' 'MALE AGE'

The terms 'LOG_{10}TIME', 'TIME OF DAY', 'ORDER', 'FEMALE AGE' and 'MALE AGE' were designated covariates.

The number of matings occurring after twenty minutes was also examined using Kendal's Test for Concordance. This nonparametric test requires the designation of a rank within a trial to each song according to the number of matings occurring. The consistency between trials with regard to these ranks is then measured. The null hypothesis is that each song is assigned a random selection of ranks, such that there is minimal agreement between trials. Kendal's Test for Concordance therefore takes into account the 'repeated measure' aspect of the experiment (i.e. that any one trial was comprised by the assay of all four songs), and is as a consequence less subject to inter-trial variability. Conflicting inferences between GLM and Kendal's Test for Concordance might therefore infer inconsistent patterns of behaviour between trials.

(1.3) RESULTS

THE EFFECTS OF SONGS ON NUMBERS OF MATINGS

The mean numbers of matings observed at each time interval for each treatment are presented in figure 1.3.1 (overleaf). (It will be seen that error bars represent the magnitude of standard errors on line graphs and standard deviations on bar charts. This reflects the output of the statistical programs used.)

The interaction between 'SONG' and 'LOG_{10}TIME' was significant; indicating that rate of copulation differed between treatments. The smallest number of matings was associated with silence. That differences between treatments remained significant when the silent control data were omitted from the analysis (table 1.3.1) inferred that the three artificial pulse songs elicited different rates of mating. All test statistics given below relate to the term 'SONG*LOG_{10}TIME'. The factor 'SONG' was never significant. Therefore, the lines were not parallel, as is expected because the number of copulations at time=0 was always 0.

TABLE 1.3.1 *Effects of simulated song on numbers of matings*

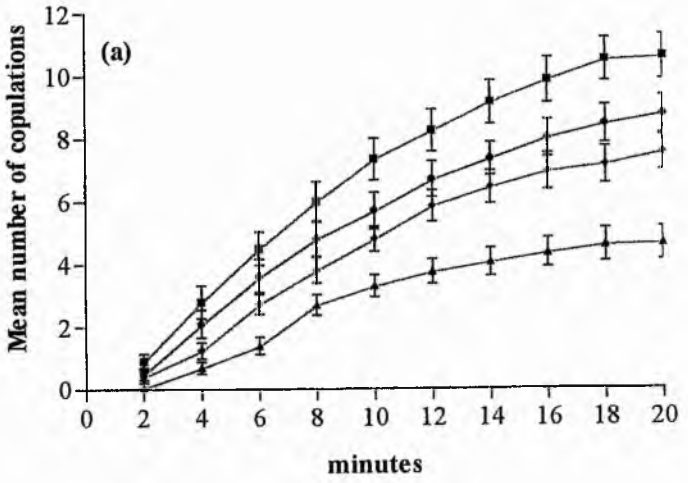
Condition	*D. melanogaster*	*D. simulans*
Silence included	$F_{3,799}$=19.42; p<0.001	$F_{3,799}$=23.77; p<0.001
Silence omitted	$F_{2,599}$=6.46; p=0.002	$F_{2,599}$=11.39; p<0.001

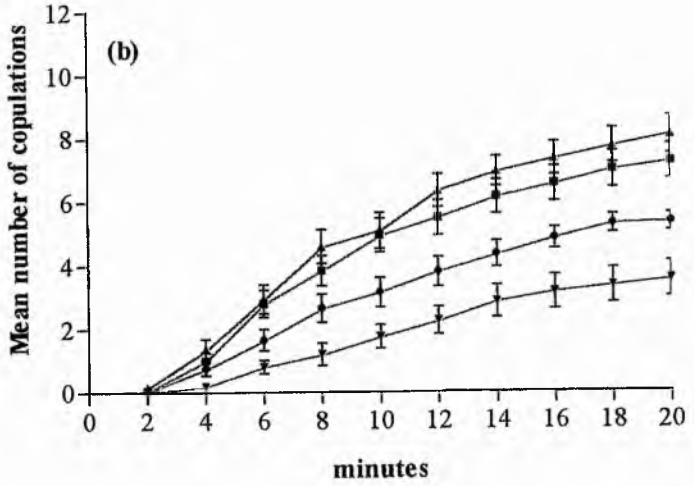
The mean numbers of copulations for each song are given in figure 1.3.2. These means are averaged over all other variables, including time. The means induced by the respective songs relative to one another differed between *D. melanogaster* and *D. simulans*, such that the greatest numbers of matings were associated with the 'conspecific' songs (figure 1.3.1). Of the three songs, the lowest number of matings occurred with 'sechellia' song for both species. This indicates that song-specific variation in sexual activity is a function of IPI differences rather than disparity in inter-burst interval between songs (figures 1.1.1 and 1.2.1; section 1.2). If inter-burst interval determined female receptivity, 'sechellia' song would have been predicted to elicit the greatest number of matings. The lower level of silence in this song corresponds to a longer total song length.

The difference in response of *D. simulans* to 'melanogaster' and 'simulans' treatments was smaller than that of *D. melanogaster* (figures 1.3.1 and 1.3.2). The latter species 'discriminates' between songs to a greater degree.

FIGURE 1.3.1 *Mean number of copulations with each song at each time interval*

(a) *D. melanogaster*; (b) *D. simulans*





Bars indicate standard errors

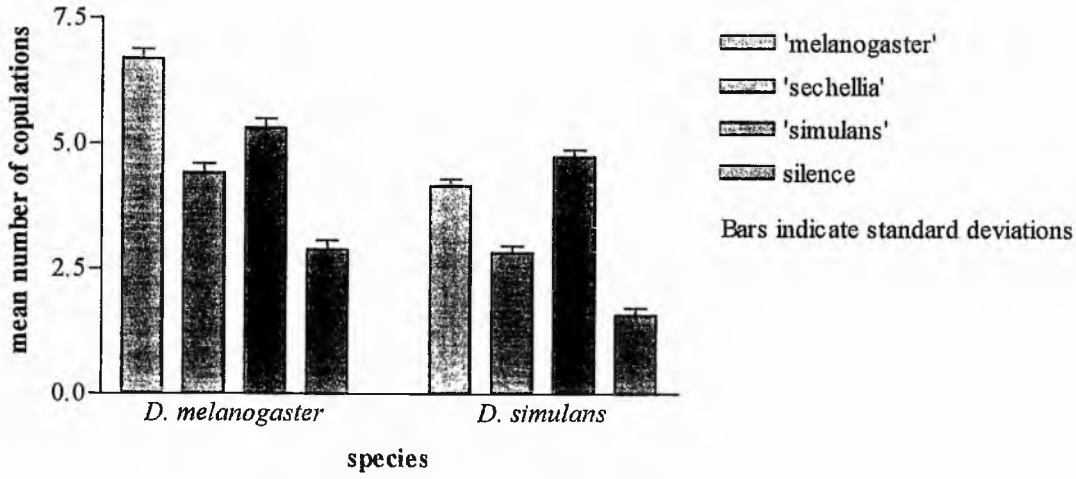
'melanogaster'

'simulans'

'sechellia'

'silence'

FIGURE 1.3.2 *Mean number of copulations with each song averaged over all variables*



THE EFFECTS OF SECONDARY VARIABLES ON NUMBERS OF MATINGS

TABLE 1.3.2 *Effects of secondary factors on numbers of matings*

Factor or covariate	*D. melanogaster*	*D. simulans*
Female age	b=0.16; s=0.060; $F_{1,799}$=6.86; p=0.009	b=-0.21; s=0.082; $F_{1,799}$=6.76; p=0.009
Male age	$F_{1,799}$=0.05; p=0.822	$F_{1,799}$=3.46; p=0.063
Time of day	b=-0.38; s=0.093; $F_{1,799}$=17.16; p<0.001	$F_{1,799}$=0.60; p=0.439
Block	$F_{3,799}$=9.08; p<0.001	$F_{2,799}$=44.85; p<0.001
Order	b=-0.34; s=0.070; $F_{1,799}$=23.42; p<0.001	$F_{1,799}$=0.22; p=0.636

b and *s* are given where the covariate has been found to have a significant effect on the numbers of matings. *b* denotes regression coefficients. The sign of *b* indicates whether the relationship is positive or negative and the magnitude of *b* gives an indication as to the gradient of the relationship. *s* is the standard deviation of the coefficient. Where the test result does not reach significance only *F* and *p* are given.

Age

NUMBERS OF MATINGS INCREASED AS FEMALES AGE

Female age influences numbers of matings in both species, but the sign of the regression coefficient differs between the two data sets (table 1.3.2). The apparent effect of age on *D. simulans* is due to four trials in which very old females were used

(aged eleven to fifteen days). If these trials are removed from the data set, female age does not covary with the number of mating ($F_{1,599}$=0.19; p=0.661). That the regression coefficient for female age given by the GLM model is positive for the *D. melanogaster* data (table 1.3.2) indicates older females are associated with a higher rate of mating. This is consistent with previous findings of both *Drosophila* (e.g. Manning 1957a; Barker 1967; Cobb *et al.* 1989) and insects in general (Manning 1957a). The disparity between the observations made here and previous findings suggesting that the sexual activity of *D. simulans* also increases with age may be due to the narrow range of ages used in the present study - two thirds of the flies were aged between eight and ten days.

THE LEVEL OF DISCRIMINATION DECREASED AS FEMALES AGE

In order to examine the effect of age on the magnitude of differences in numbers of matings between songs, the *D. melanogaster* data were divided into two groups according to female age, one comprising trials carried out on flies aged between two and four days, and the other comprising flies aged between five and eight days. The two groups were of approximately the same size. Differences in numbers of matings between 'melanogaster' and 'simulans' song were found for the 'young' group (i.e. the 'SONG* LOG_{10} TIME' term was significant: $F_{1,219}$=5.14, p=0.024) but not the 'old' group ($F_{1,179}$=0.38, p=0.536). Older females would seem to be less sensitive to differences between pulse songs.

Male age did not influence numbers of matings (table 1.3.2).

Time of day

Whereas the overall numbers of matings of *D. simulans* was independent of time of day, the effect of time of day was significant for *D. melanogaster*. The regression coefficient is negative (table 1.3.2), indicating that the level of sexual activity of *D. melanogaster* decreased throughout the day. This is consistent with findings of declining locomotion throughout the day in *D. melanogaster* (Konopka and Benzer 1971), but contradicts the observations of Cobb *et al.* (1987) who found that courtship latency and duration of *D. simulans* increase throughout the day whereas and those of *D. melanogaster* are independent of time of day. The higher rate of mating induced by the use of older *D. simulans* females may have masked any effect of time of day.

Block

'Block' was a significant factor in determining rate of copulation in both trials (table 1.3.2). This might indicate effects of bottle cultures on mating behaviour, or inter-trial variability in external influences. The latter of these suggestions is supported by the non-significance of a repeated measures test reported below. Furthermore, on some days, the rate of copulation did not differ between songs (personal observation). The general level of activity in the mating chamber varied a great deal between days on which experiments were carried out, and the rate of copulation seemed lower when this agitation was higher. These personal observations are consistent with the findings of Robertson (1982), who found that courtship latency was positively correlated with the level of locomotion. Whichever factor is responsible for differences in the level of agitation of flies, which must be extrinsic to the experimental procedure, is also likely to account for the variation in rate of mating between trials. Given the relationship between mating speed and level of variation between songs found above, it is therefore advisable to execute a large number of mating trials before conclusions are drawn.

Order

Unexpectedly, the order in which treatments had been assayed affected the rate of copulation for *D. melanogaster*. This effect is attributable to a deficiency of matings associated with the fourth song, which was due to a bias in the experimental design – an excess of silent treatments in the fourth position. As silence is associated with a lower rate of copulation than pulse songs, this can account for this result. If the fourth song is removed from the data set, the effect of order on numbers of matings is no longer significant ($F_{1,599}$=2.79, p=0.096).

NONPARAMETRIC ANALYSIS

The ranking of number of matings after twenty minutes was in agreement between trials (table 1.3.3), indicating consistency between trials. The mean number of copulations after twenty minutes followed the same trends as the rates: 'conspecific' song elicited the greatest number of copulations, with 'sechellia' song having the lowest number of the three songs and silence the smallest quantity of all (figures 1.3.1 and 1.3.3).

The omission of silence from the analysis produced significant results for the *D. simulans* data but not the *D. melanogaster* data, although the test was nearly significant (table 1.3.3). This points to some disagreement in ranking between trials; the effect on numbers of matings of the songs relative to one another was not always consistent.

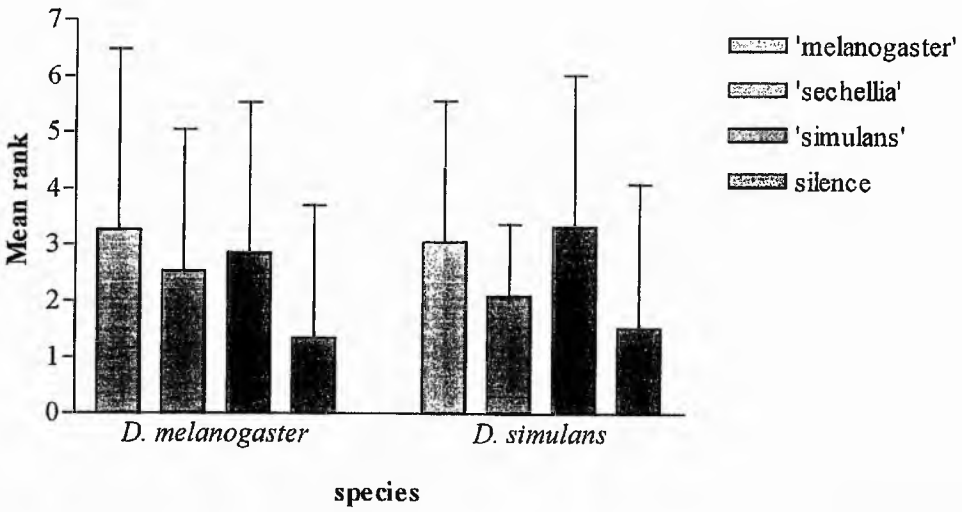
TABLE 1.3.3 *Nonparametric analysis of number of matings after twenty minutes*

Condition	*D. simulans*	*D. melanogaster*
Silence included	W=0.44, χ^2_3= 26.31, p<0.0001	W=0.42, χ^2_3 = 25.45, p<0.0001
Silence omitted	W=0.37, χ^2_2= 14.68, p=0.0007	W=0.14, χ^2_2= 5.55, p=0.062

W represents Kendal's test statistic, which gives a magnitude of chi-square.

FIGURE 1.3.3 *Mean ranks of songs*

Bars represent standard deviations



(1.4) DISCUSSION

The following have been observed:

- The rate of mating of females increased when they were subjected to artificial pulse song.
- Rate of mating differs according to the mean IPI and IPI cycle length of a pulse song.
- Rates of mating elicited by the different songs relative to each another differed between species, such that the highest rates were observed with 'conspecific' pulse songs.
- Overall rates of mating and differences between songs in rates of mating were greater in *D. melanogaster* than in *D. simulans.*

These findings will now be considered in terms of pre-mating isolation in the laboratory and in the field, and on the evolutionary forces shaping courtship song in *Drosophila.* First, the validity of these observations will be considered. Then a role for pulse song in mate-recognition and sexual isolation will be discussed. Finally, the species mate-recognition systems of *D. melanogaster* and *D. simulans* will be compared.

EFFECTS OF THE ARTIFICIAL ENVIRONMENT ON SEXUAL BEHAVIOUR

The experimental apparatus was far removed from the natural environment of fruit flies. Various disparities might alter both male and female mating behaviours. For example, the limited space of the mating chamber might reduce the capacity of females to reject courting males, given that the most effective means of rejection in the field is decamping (Speith 1974; Gromko and Markow 1993). Ewing (1983) observed that courtships in confined conditions were much longer than those observed in larger chambers. Cobb *et al.* (1989) found that the rate of copulation of *D. sechellia* increases in the presence of an available food source. Females in the field are much less active than in the laboratory (Partridge *et al.* 1987), indicating the level of sexual excitation to be higher in the natural environment (Robertson 1982; Cobb *et al.*_1987). Virgin males were used to standardise prior sexual encounters. However, as the vigour of male courtship increases with experience (Manning 1959a), this standardises courtship to low intensity.

Only a small number of females in the field are sexually receptive at any one time (Speith 1974; Gromko and Markow 1993). A receptive female is therefore likely to find herself courted by many males over short periods of time. The equal ratio of males and females used in the present study may therefore be rather artificial. Sex ratio influences the frequency of hybridisation in the laboratory, probably as a result of differences in male behaviour (section 1.1.1). Furthermore, there is some evidence that females courted by more than one male are

more discriminating. Hoikkala and Aspi (1993) found that females of the Drosophila virilis group tended to accept a male producing abnormal courtship song (as a result of his wings having been shortened) if he was the only courter, but reject him in favour of a winged male if this alternative was available to her. Wu *et al.* (1995) found if a sexually isolated strain of *D. melanogaster* was confined with males of both the same strain and 'foreign' strains, the rate of inter-strain copulation was lower than that found with foreign males alone. The mating success of males seems to depend on relative criteria in these instances; females compare and choose males if circumstances permit. The increase in female receptivity by artificial courtship song persists for a few minutes following stimulation (Schilcher 1976b; Kyriacou and Hall 1984), indicating that females 'remember' courtship songs, in which case they might be able to compare them. In the present study, females were kept in single-sex vials from a very young age, and the experiment did not present them with any means to compare songs.

Experimental apparatus and treatment of flies may well have affected sexual behaviour. However, it is difficult to imagine the observed differences in mating speed between songs as artefacts of the protocol. Moreover, disparities between experimental and field conditions are likely to have reduced rather than magnified female preferences between songs.

THE ROLE OF SONG IN MATE-RECOGNITION AND SEXUAL ISOLATION

Mating speed of flies was increased by exposure to pulse song, in agreement with previous findings cited in section 1.1.3. This is unlikely to be a function only of its effect on male behaviour. The element of mate-recognition missing when wingless males court females has been at least partially restored: female sexual receptivity was increased by pulse song. Previous findings show that the rate of copulation associated with unmodified males is similar to that observed when the courtship of wingless *D. melanogaster* males is supplemented with artificial pulse song (Kyriacou and Hall 1982); the reduction in the rate of copulation caused by the removal of wings was entirely a function of the absence of pulse song and not other stimuli provided by male wing display. Pulse song would appear to be a major component of the mate-recognition system.

The effectiveness of a song in stimulating a female depended on her species, such that mating speeds were maximum for conspecific pulse songs: pulse song is a species-specific mate-recognition signal. These data clearly suggest a mechanism by which female *D. melanogaster* and *D. simulans* distinguish between males of difference species. Preference

for conspecific pulse song may therefore contribute to sexual isolation occurring in laboratory cultures and the field.

RESPONSE TO CRITICISM OF PULSE SONG AS A MEANS OF SPECIES-RECOGNITION

Noor and Aquadro (1998) suggest that mean IPI is unlikely to contribute towards sexual isolation because it is highly malleable in response to environmental change. The influence of temperature on IPI in *D. melanogaster* is the most thoroughly documented of these effects: mean IPI decreases linearly with increase in temperature (Shorey 1962; Ritchie and Kyriacou 1994). However, the preferred mean IPI of females is also negatively correlated with temperature (M. G. Ritchie, pers. comm.), such that preference remains attuned to trait regardless of fluctuation in temperature. Before other 'environmental effects' are given as evidence against a role for IPI in species-isolation, they should be defined more clearly and tested for influence over female preference for IPI. It may be found that environmental changes tend to affect female preference in directions that mirror effects on IPI, as is the case with temperature. This coupling of species-specific signal and preference would more reasonably constitute evidence in favour of IPI as a species-recognition signal than evidence against such a function. Likewise, changes in mean IPI of long-term laboratory cultures, also cited as evidence against IPI in species-isolation by Noor and Aquadro (1998), are not necessarily divorced from correlated changes in female preference. Greenacre *et al.* (1993) found that female preference for IPI cycle length in bottle cultures of *D. melanogaster* seemed to evolve in such a direction that it matched the male signal.

Noor and Aquadro (1998) also argued that there is more variation in mean IPI between populations than the majority of measurements infer (Ritchie *et al.* 1998). This is not incongruent with a role in species-recognition, assuming there is no gene flow between populations (Iwasa and Pomiankowski 1995). Rather, the sexual isolation of *D. melanogaster* populations of divergent mean IPI (Wu *et al.* 1995; Pugh 1997) constitutes evidence in favour of this role.

Direct measures of female preference for pulse song are required to support or refute its supposed contribution towards species isolation. Noor and Aquadro (1998) compared the pulse songs of backcross *D. persimilis/D. pseudoobscura* males that were successful in mating to those unsuccessful. As there was no difference in mean IPI between the two groups, it was concluded that IPI was not a criterion by which females discern between the species, although females mate assortatively and the two species differ in mean IPI. However, as it was pointed out earlier (1.1.2), a danger in using real males to investigate mate-preference is that that the song trait under investigation may not be independent of other traits correlated with mating success, perhaps amplitude or quantity of courtship song, or mating vigour. Playback experiments, which avoid these confounding possibilities, have not

been carried out with these species. Even if mean IPI is not a species mate-recognition signal in species of *Drosophila* outside of the melanogaster complex, the findings of the present study are not necessarily challenged. The particular elements facilitating recognition of conspecifics may differ between systems; mate-recognition systems may be species-specific, as is discussed later.

IS FEMALE PREFERENCE FOR CONSPECIFIC PULSE SONG A CAUSE OR EFFECT OF REPRODUCTIVE ISOLATION?

Copulation of *D. melanogaster* and *D. simulans* individuals is maladaptive (Lemeunier *et al.* 1986). As males seem to court females independently of species in sympatric populations (section 1.1.1), the preference observed here might reflect selection favouring females who discriminate against heterospecific males. However, differences in species mate-recognition system may arise without selection arising from heterospecific interactions. Can the data gathered in the present study be seen to support one or other of these hypotheses?

An adaptive preference?

Differences in mating speed between songs decreased with age. The majority of females collected from field populations have been inseminated (Speith 1974; Gromko and Markow 1993), presumably soon after eclosion, so the effects of age are unlikely to be subject to selective pressure with respect to female receptivity. However, this behaviour could be adaptive if it evolved in low-density populations, in which opportunities for mating are likely to arise infrequently. If so, that virgin females become less discriminating as they age could reflect females balancing the benefits of choosing between males against the risk of not being inseminated at all. This line of thinking suggests that female preference for pulse song evolved to permit differentiation between males. Although this differentiation is not necessarily between males of different species, there is as yet no evidence for intraspecific sexual selection on IPI.

A broad preference and its implications

Mating speeds elicited by *all* pulse songs were greater than those of the silent controls; females are stimulated most efficiently by conspecific pulse song, but are also stimulated, to lesser degrees, by song of greater or lesser IPI and cycle length. This has been found previously in *D. melanogaster* for IPI (Bennet-Clark and Ewing 1969; Schilcher 1976b;

Kyriacou and Hall 1982) and IPI cycle length (Greenacre *et al.* 1993). These data are dissimilar to those obtained through a similar study of *D. biauraria*, in which artificial pulse song of heterospecific IPI *lowered* the rate of copulation below that observed for silence and elicited rejection responses from females (Tomaru *et al.* 1995). A broad range of pulse songs qualify as mating signals for *D. melanogaster* and *D. simulans* in contrast to the narrow range stimulating *D. biauraria*. The breadth of a preference is likely to affect the strength of pre-mating isolation. For example, the narrow preference of *D. biauraria* females correlates with a high degree of sexual isolation in the laboratory (Tomaru *et al.* 1995). In turn, a low degree of sexual isolation observed in the laboratory has a bearing on the frequency of hybridisation in the field: strains of *D. melanogaster* and *D. simulans* collected from a sympatric population of an unusually high frequency of hybrids exhibited a relatively low measure of sexual isolation under laboratory conditions (Sperlich 1962).

A preference 'designed' to reject heterospecific copulation might be expected to be more specific than has been observed here. Rather, it may be more accurate and productive to think of the lower mating success of heterospecific males projected by these data as a consequence of the lower efficiency of their pulse song in stimulating females. Females of both species were least receptive to the song of *D. sechellia*, a species that they would never encounter.

To summarise, differences in mating speed between pulse songs and the effect of age may indicate species-specific female preference for conspecific pulse song, and the observed differences could well account for the low level of hybridisation reported from the laboratory and field. However, the broadness of the preference and low mating speed of 'sechellia' song infer that heterospecific interactions in sympatry have not been of primary importance in shaping pulse song receivership in these species. Sexual isolation is a consequence rather than a cause of divergence in pulse song, in agreement with Paterson (1980): 'Speciation is said to have occurred when the SMRS [species mate-recognition system] of the members of the daughter population has been so extensively modified that it no longer functions effectively with members of the parental or any other population.'

COMPARISON OF THE MATING SYSTEMS OF D. MELANOGASTER *AND* D. SIMULANS

The mating speeds of *D. melanogaster* were higher than those of *D. simulans*. The rate of copulation observed in silence was similar between species, and is well-established that *D. simulans* has a longer latency or duration of courtship than *D. melanogaster* under a variety of laboratory conditions (e.g. Manning 1959b; Cobb *et al.* 1987), so it is unlikely that these

differences reflect a greater suitability of *D. melanogaster* to this type of assay. There are other differences in the sexual behaviour of the two species. For example, *D. melanogaster* males court in darkness, but courtship of *D. simulans* is light dependent (Hardeland 1972; Speith 1974; Cobb *et al.* 1989), and the latter species also has larger eyes. Whereas *D. simulans* will court only females bearing conspecific cuticular hydrocarbons, regardless of whether they are conspecific or heterospecific, alive or dead, the courtship of *D. melanogaster* males is non-specific with respect to pheromones (Coyne *et al.* 1994). Here, differences in mating speed between songs were greater for *D. melanogaster* than for *D. simulans* (although this may reflect the greater age of the latter species). Perhaps these differences reflect primary importance of auditory stimuli in the mate-recognition system of *D. melanogaster*, whereas *D. simulans* place more emphasis on chemical and visual cues. Kawanishi and Watanabe (1980) suggest that the high variability in mean IPI between strains of *D. simulans* relative to *D. melanogaster* reflects a greater importance of song in the latter species. The mating system of *D. sechellia* may rely on different stimuli again. Male courtship of this species is independent of species pheromone profile (Cobb and Jallon 1990) and dependent on behavioural cues, as males do not court dead females (Cobb *et al.* 1989; Coyne and Charlesworth 1997), in contrast to *D. melanogaster* and *D. simulans* (Bastock 1956; Coyne *et al.* 1994). Mating systems are species-specific and findings from one species cannot necessarily be extrapolated to another, even when the two are closely related. Perhaps the difference between *D. melanogaster* and *D. simulans* in reliance on auditory stimuli relative to behavioural or pheromonal cues circumvented a 'need' for reinforcement, or is even itself an effect of reinforcement.

CONCLUDING REMARKS

Female preference for conspecific pulse song has been argued to be responsible for the ethological isolation of *D. melanogaster, D. sechellia* and *D. simulans* observed in the laboratory, and at least a contributory factor towards the low level of hybridisation in sympatric populations of *D. melanogaster* and *D. simulans*. The data do not lend support to suggestions that reinforcement has caused divergence of IPI in these species. Differences in the pulse songs of these species are likely to be a cause rather than an effect of reproductive isolation seeing as preferences for conspecific songs appear broad and females do not discriminate as such against the songs of sympatric species.

IDEAS FOR FURTHER STUDIES

Relative choice

The effect of permitting females relative choice could be investigated in various ways (e.g. Aspi and Hoikkala 1993; Wu *et al.* 1995). Whether short-term experience of

songs influences female preference could be investigated by 'priming' females with either heterospecific song or conspecific song, then testing their mating speed with either heterospecific song or conspecific song.

Heterospecific courtship

A playback experiment could be carried out using *D. melanogaster* males to court females of both species, given that male *D. melanogaster* court *D. simulans* as they do their own species (e.g. Wood and Ringo 1980). This could indicate the extent to which the differences in behaviour of *D. melanogaster* and *D. simulans* females is a function of male courtship intensity (*D. melanogaster* males court with a greater intensity than males of *D. simulans*), and infer roles of other species-specific cues in courtship.

Intra-specific sexual selection

It has been argued that intraspecific processes are more important than heterospecific interactions in shaping pulse song. Therefore, perhaps attention should be turned towards intraspecific sexual selection. For example, a narrower range of songs could be tested by playback, using single pairs of flies rather than groups of 20 to make more sensitive the measurement of preference, as has been achieved by Ritchie *et al.* (1998) with *D. montana*.

Field observation

It is important to establish, via direct observation, how much interspecific courtship and copulation actually occurs in the field. For example, much investigation has been made into the role of pheromones in male discrimination between species, yet observation of *D. melanogaster* and *D. simulans* in their natural environments infers that males court females irrespective of species.

Mean IPI differences in sympatry and allopatry

If reinforcement is responsible for short-term divergence of mating signals in *D. melanogaster* and *D. simulans*, IPI differences between the two species would be more defined in individuals from sympatric populations than in individuals taken from allopatric populations.

Comparison of mating systems

Why is pre-mating isolation more severe in *D. biauraria* than *D. melanogaster* and *D. simulans*? It would be interesting to compare the natural environment of the species with a view to the identification of differences leading to this.

Chapter Two

Stimulation of female *Drosophila melanogaster* by pulse song rhythms

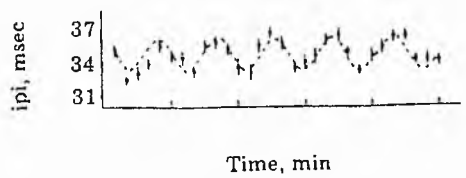
(2.1.1) A REVIEW OF THE LITERATURE

The interpulse-interval (IPI) of courtship song fluctuates rhythmically in some species of the melanogaster subgroup, including *D. melanogaster* (Kyriacou and Hall 1980; Alt *et al.* 1998; figure 2.1.1.1), *D. simulans* (Kyriacou and Hall 1980), *D. sechellia* and *D. yakuba* (C. P. Kyriacou, pers. comm.). The duration of a complete oscillation differs between species to a greater extent than it varies within species (Kyriacou and Hall 1980; section 1.1.1). The length of an IPI cycle of *D. melanogaster* pulse song has been estimated at approximately 55 seconds by Kyriacou and Hall (1980) and 68 seconds by Alt *et al.* (1998).

ARTIFICIAL CYCLING PULSE SONG INCREASES FEMALE RECEPTIVITY

The low mating success of wingless males, which are unable to produce song, can be increased when supplemented by synthetic pulse song, as described in chapter one. If the IPI of a pulse song oscillates as described above, mating speed is greater than if song is of invariant IPI. Furthermore, the period of an IPI cycle determines its influence over female receptivity: maximum mating speeds are observed with pulse songs of conspecific IPI cycle length. Specifically, of the periods tested on mating speed, song of conspecific or longer oscillation period stimulate female *D. melanogaster*, but shortened cycles do not. *D. simulans* females show the opposite: lengthened song cycles have lesser effects on receptivity than song of conspecific period (Kyriacou and Hall 1982, 1984; Greenacre *et al.* 1993). As the song characteristic of *D. simulans* has a shorter period than that of *D. melanogaster*, this might reflect some element of species-discrimination in preference for IPI cycle length. Synthetic pulse songs of conspecific cycle length superimposed over heterospecific mean IPI have little effect on female receptivity (Kyriacou and Hall 1982), indicating that it is the interaction between mean IPI and periodicity, rather than IPI gradients, that is a song's critical feature.

FIGURE 2.1.1.1 *Oscillating pulse song of* D. melanogaster
(reproduced from Kyriacou and Hall 1980)



Which features of artificial song of conspecific period cause it to stimulate females where songs of invariant IPI and heterospecific period do not? A first consideration is the length of time taken for a signal to affect females. Some authors have expressed doubt as to whether oscillation can be perceived by females in the field on the basis that the duration of courtship is a few seconds, whereas a complete cycle of IPIs lasts for around a minute in *D. melanogaster* (e.g. Ewing 1983; Alt *et al.* 1998) (although there is no reason to suppose that females require a whole cycle of song). A typical *D. melanogaster* courtship in the laboratory lasts for a few minutes. However, as was discussed in the previous chapter, courtships occurring under artificial conditions may be longer than those occurring in the field for various reasons. Information based on field observation is lacking. It is commonly supposed that all courtships in the field last for only a few seconds, and that signals must be received on this time-scale. However, this view might wrongly assume that mating behaviour is simpler and more homogeneous than it actually is. First, it will be argued that the length of time for which a female is courted may depend on whether she is accessible or inaccessible to courtship. Secondly, differences between signals that 'summate' and signals which 'trigger' sexual excitation will be discussed.

COURTSHIP DURATION OF ACCESSIBLE AND INACCESSIBLE FEMALES

The length of the majority of *D. melanogaster* courtships in the field is of the order of 6 to 20 seconds (Partridge *et al.* 1987; Gromko and Markow 1993). However, no distinction has ever been drawn between courtship of receptive and unreceptive females. It is conceivable that courtships of the two groups differ in length, as the laboratory and field observations described below may be taken to show.

First, courtships occurring in the field only rarely end in copulation (Partridge *et al.* 1987; Gromko and Markow 1993), and no complete courtship – that is, from initial encounter to copulation – of *D. melanogaster* seems to have been recorded in the literature. Second, the majority of females in the field are likely to be unreceptive. Boulétreau (1987) estimated that 20 per cent of females captured from a field population were younger than 24 hours old, and typically between 85 and 96 per cent of females captured in the field are carrying sperm (Boulétreau 1978, Gromko and Markow 1993). The sexual receptivity of both these groups (sexually immature and inseminated females) is low, and they are likely to reject sexual advances of males (Manning 1967a; Ewing and Ewing 1987; Gromko and Markow 1993). Ewing and Ewing (1987) recorded that inseminated females extrude nearly continuously while feeding if males are present. Inseminated females often remate, but not until the

sperm stored from the previous mating has been diminished (Manning 1967a; Gromko and Markow 1993).

Third, males will court inseminated females (Ewing and Ewing 1987; Gromko and Markow 1993), although the attractiveness of a female decreases in the short term after her being mated as a consequence of inhibitory substances transferred from the male (Bastock and Manning 1955; Scott and Jackson 1990). It is possible that the small chambers used in the laboratory have led to overestimation of the inhibitory effects of anti-aphrodisiac compounds in the field (Ewing and Ewing 1987). Attractiveness to males returns before females regain sexual receptivity. Female behaviour and not male behaviour (Partridge et al. 1987; Gromko and Markow 1993) terminate most courtships. Finally, when observed in large mating chambers and in the field, inseminated females received a great many more bouts of courtship lasting under five seconds than virgin females (Ewing and Ewing 1987).

It is therefore possible that the majority of the courtships observed in the field are terminated by unreceptive females, which reject any courting male, independently of the *content* of his courtship; they are not 'accessible' to stimulation (Manning 1967a). The length of courtship experienced by receptive females, whether it is ultimately unsuccessful and successful, is not necessarily of the same length, and could be longer than this. *Some* field courtships last for several minutes (Gromko and Markow 1993). The evidence that female *D. melanogaster* distinguish between males within a few seconds is therefore inconclusive.

Observations made by M. A. F. Noor of *D. persimilis* and *D. pseudoobscura* in the field indicate that courtships ending in copulation are shorter than unsuccessful courtships (M. G. Ritchie, pers. comm.). However, there are major differences between these species and *D. melanogaster* in terms of sexual behaviour. For example, aggressive interaction between males is very frequent in *D. pseudoobscura,* whereas male-male competition is of little importance in *D. melanogaster* (Partridge *et al.* 1987; Gromko and Markow 1993). Species-specificity of mean IPI may be less important for mate-recognition in these species, and IPI oscillation reported for *D. persimilis*, at around a second for a complete cycle, is of a different scale (Noor and Aquadro 1998). Therefore, in the context of the current interests, field observations of these species cannot necessarily be extrapolated directly to *D. melanogaster.*

SIGNALS CAN SUMMATE OR TRIGGER FEMALE SEXUAL EXCITATION

If a female is accessible to courtship, for how long does she require stimulation before she becomes ready to mate? It has been assumed implicitly so far that the receptivity of a female is stimulated by the courtship of a single male. However, courtship stimuli do not simply release sexual excitation. Rather, the stimuli a male directs at a female accumulate until a critical level is reached (Bastock and Manning 1955; Manning 1967a; Speith 1974). Sexual excitation resulting from stimulation by artificial pulse song remains at a raised level for a few minutes before declining (Schilcher 1976b; Kyriacou and Hall 1984). Natural populations are at high density and a receptive female is likely to be courted by many males within short spaces of time (Speith 1974). Therefore, the stimuli provided by more than one courtship could contribute to a raised level of excitation, and the male that eventually mates with a female may not have been solely responsible for her arousal. Accordingly, Speith (1974) noted that females do not tend to respond positively to the first bout of courtship they experience - typically, a female was courted two or more times before accepting a male.

Therefore, even if field courtships are as brief as is commonly assumed, the length of time required for a signal to take its effect on females may be a few minutes. It is possible that the long duration of courtship observed in the laboratory reflect the time required for pulse song to be perceived as a mate-recognition signal after all. Females in the laboratory tend to be highly agitated in comparison with those in the field, which is associated with low sexual receptivity (Robertson 1982, Ewing and Ewing 1987; Partridge *et al.* 1987). Therefore, the time taken to induce mating in laboratory females may reflect the quantity of stimulation required from a basal level of stimulation, whereas a typical female of a dense field population may be partially aroused for much of the time, and therefore require a shorter 'final' courtship.

Here, a distinction can be made between mating stimuli that summate, as described above, and mating stimuli that release sexual excitation. This latter type of signal theoretically translates an internal level of excitation into a positive physical response. A female may be highly excited, but will not mate until she perceives a particular signal, a 'trigger'. A mating signal that triggers female receptivity is expected to be brief, if females exercise any form of preference between males. A fully receptive female is susceptible to mating with any willing male in the immediate vicinity, not just the one responsible for the mating signal. There is some indication that conspecific pulse song acts as a trigger whereas sine song (section 1.1.1), can only summate female excitation (Schilcher 1976b; Kyriacou and Hall 1982, 1984).

Schilcher (1976b) noted that copulation in *D. melanogaster* is almost invariably preceded by a bout of pulse song. Sine song does not differ between species of the melanogaster subgroup (Cobb *et al.* 1989).

The argument has returned full circle: the length of time required for cycling pulse song to stimulate females is predicted to be brief. However, relevant theoretical distinctions have been drawn between the courtship of accessible and inaccessible females, and between signals that summate and trigger sexual excitation.

WHAT'S IN A SONG?

Which properties of cycling song are likely to be responsible for stimulating females, bearing in mind the signal may take its effect in only a brief period of time? One feature of rhythmic song is its constant oscillation of IPI. This occurs at a rate specific to species, presenting a possible short-term criterion of female selection between species. Whereas there is some overlap between *D. melanogaster* and *D. simulans* in 'discrete' IPIs (Kyriacou and Hall 1980), there is less similarity between the songs of the species in terms of rates of change. A preference based on IPI gradients may then be more discerning than one reliant on 'counting' IPIs of particular lengths. However, the likelihood that females are able to detect whether a male's IPI is cycling is lessened by the high level of variation of IPI within songs (Kyriacou and Hall 1980; Ewing 1983, 1988; Crossley 1988; Alt *et al.* 1998). This is particularly true if courtships are brief, as a specific gradient of IPI is unlikely to be perceptible within a short space of time if there is a great deal of background variation. In addition, courtships usually contain intermittent bursts of song rather than continuous signalling. A representation of a *D. melanogaster* pulse song recorded during a typical courtship in the laboratory is given in figure 2.1.1.2 (overleaf). Clearly, there is a great deal of variation, and bouts of song are short and irregular, such that it is not possible to discern by eye the periodic cycling of IPI. Furthermore, courtships in the laboratory may be more intense than those occurring in the field.

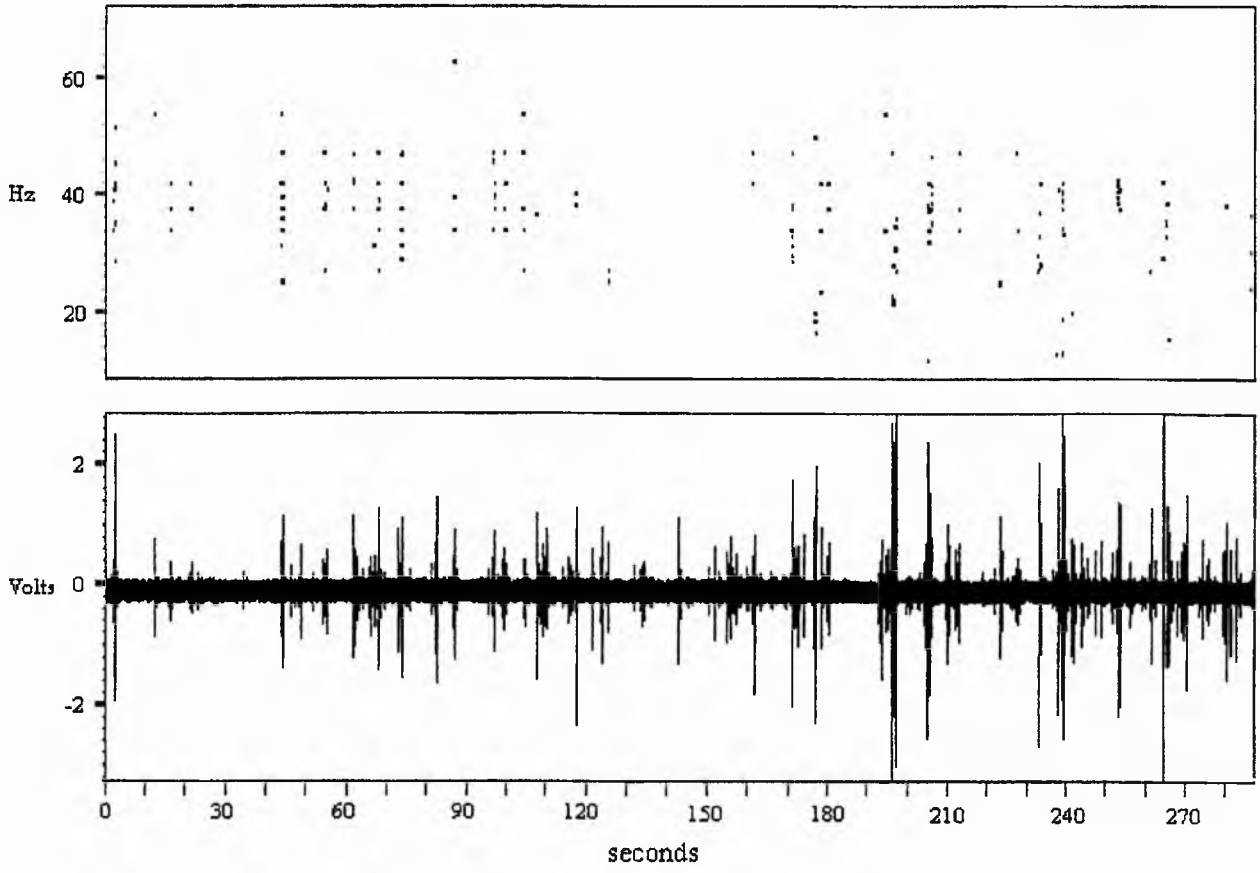
Perhaps, then, it is the increased variation of IPI generated by oscillation that is its salient feature. A preference for cycling of IPI about a species mean would be apparent if, say, females require a range of IPIs about a species-specific value. Alternatively, an IPI of a particular length most effective in stimulating one female might be ineffective in stimulating another, such that there is variation between females within a species. This could arise if individual females have different fixed preferred mean IPIs or if an individual's favoured IPI fluctuates temporally. These preferences

would be manifest as the wide range of pulse songs found to stimulate females in chapter one and other studies (section 1.4), as receptivity was measured in groups as opposed to individual females.

To summarise, playback experiments indicate that the IPI cycle of pulse song acts as a species-specific mate-recognition signal in *D. melanogaster*. Speculation has been made as to the property of oscillating song that gives it this property. That the IPI of real pulse song is highly variable makes it likely that the increased variation of IPI in periodic song is its salient feature, particularly as it may take effect in only a short space of time. This hypothesis will be tested using playback of artificial pulse song.

FIGURE 2.1.1.2 *The song of a typical* D. melanogaster *courtship*

The lower portion of the figure is a sonogram of an individual male's courtship song over 280 seconds, with the vertical axis indicating the amplitude of the pulses. The upper portion plots IPI in Hz over the same horizontal axis. There is no obvious methodical oscillation of IPI as can be seen in figure 2.1.1.1, which uses mean IPI



(2.1.2) EXPERIMENTAL AIMS AND RATIONALE

Which properties of artificial cycling song increase female sexual receptivity? One possibility is the increased variation in IPI produced by oscillation is its important feature. If so, a song that varies to the same degree as cycling song will stimulate females as cycling song does. Playback is an ideal technique to test this hypothesis, as the increased variation can be tested independently of cycling. This was carried by constructing an artificial song of distribution of IPIs identical to that of a rhythmic song, but delivered in a random rather than sinusoidal sequence. The effect of this novel song on mating speed was measured and compared with the effect of artificial patterned song. In addition, mating speeds associated with an artificial song of invariant IPI and with silence were recorded.

The species tested was *D. melanogaster*, and the characteristics of artificial pulse songs were tailored accordingly: the mean IPI of all songs was 34 milliseconds and the IPI cycle length of cycling song was 55 seconds (section 1.1.1). The four treatments tested for effects on female receptivity were:

- 'cycling' song of period 55 seconds
- 'random' song in which IPI varies to the same degree as 'cycling' but non-rhythmically.
- 'constant' pulse song of invariant IPI
- silence

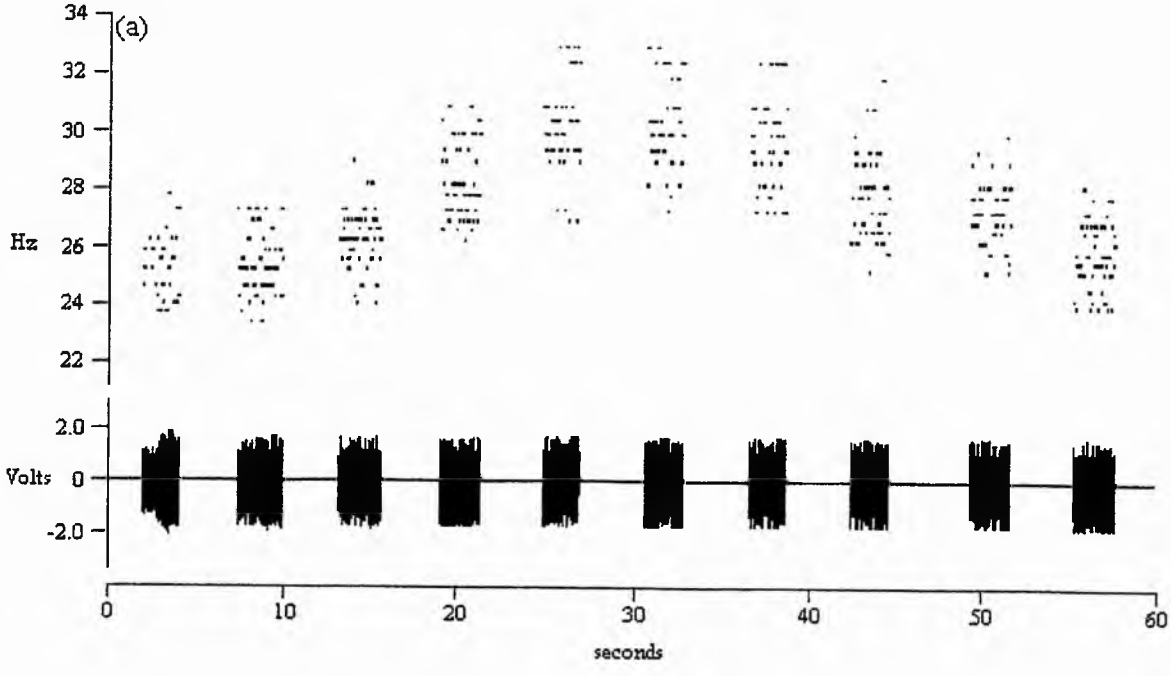
Measurement of female sexual receptivity and experimental design was as described in section 1.1.2.

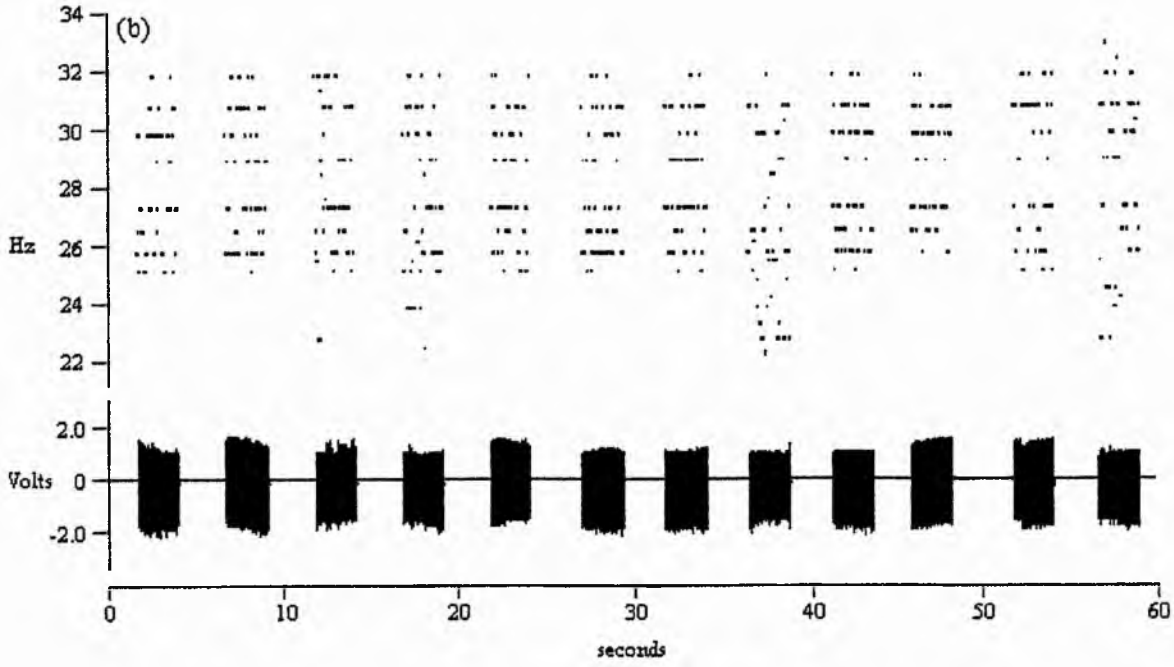
(2.2) MATERIALS AND METHODS

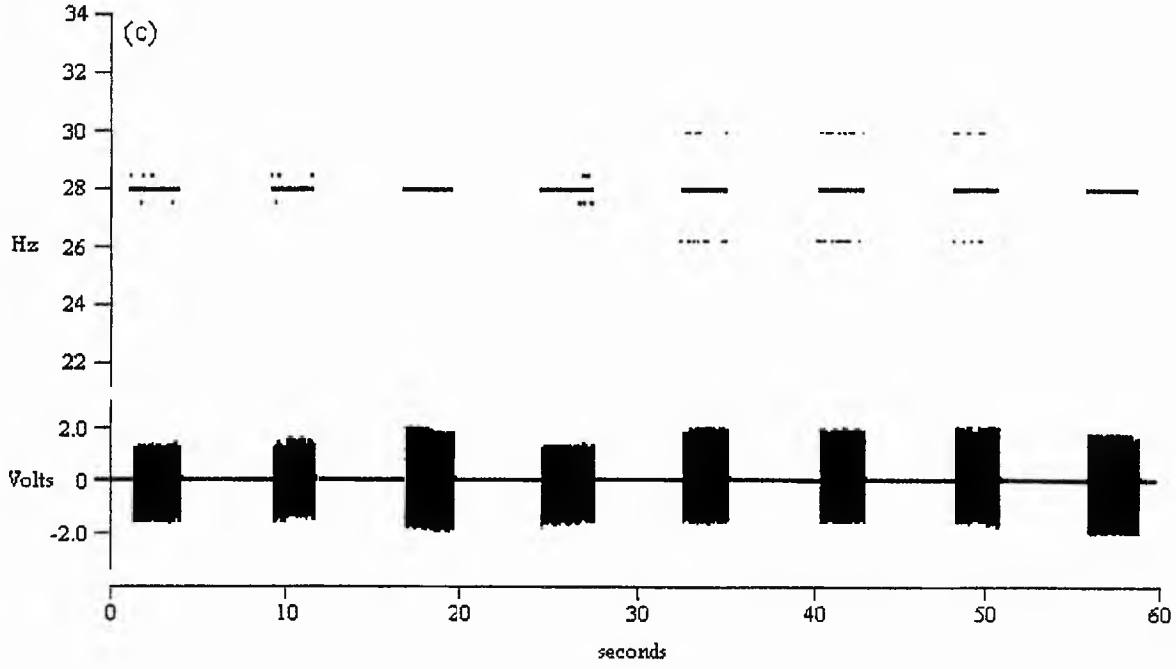
Experimental stocks, protocol, song synthesis and statistical analysis were the same as those described in section 1.2. The two experiments differed only in the characteristics of the four artificial songs assayed and in that only *D. melanogaster* was tested here.

The mean IPI of all three songs was 34 milliseconds. The IPI of 'cycling' song incorporated a 55-second *D. melanogaster*-like period, with amplitude +/-3.4 milliseconds, such that it was similar to the 'melanogaster' song of the previous experiments (section 1.2). The IPIs of 'random' and 'constant' did not cycle. 'random' song was constructed from the distribution of IPIs constituting 'cycling', but in a random rather than a sinusoidal sequence, such that the level of variation was identical between the two songs (figure 2.2). Random 'noise' was added to each song as in chapter one, and this was the only variation in the IPI of 'constant' song. As before, the rate of mating was also observed in silence.

FIGURE 2.2 *The three artificial songs*
(a) 'cycling'; (overleaf) (b) 'random'; (c) 'constant'




(b)
34
32
30
28
Hz 26
24
22
2.0
Volts 0
-2.0
0
10
20
30
40
50
60
seconds


(c)
34
32
30
28
Hz 26
24
22
2.0
Volts 0
-2.0
0
10
20
30
40
50
60
seconds

(2.3) RESULTS

THE EFFECTS OF SONGS ON NUMBERS OF MATINGS

The mean numbers of matings observed at each time interval for each treatment are presented in figure 2.3.1. As in section 1.3, all test statistics refer to the interaction between 'SONG' and 'LOG_{10}TIME'. When all four songs are incorporated into the analysis differences between songs are significant. However, when 'cycling' song is omitted, numbers of matings do not differ between treatments (table 2.3.1). Therefore, of the three pulse songs, only 'cycling' song increases sexual activity over that observed in silence.

TABLE 2.3.1 *Effects of simulated song on numbers of matings*

Condition	Test result
'cycling' included	$F_{3,839}=17.90$; $p<0.001$
'cycling' omitted	$F_{2,629}=2.71$; $p=0.665$

THE EFFECTS OF SECONDARY VARIABLES ON NUMBERS OF MATINGS

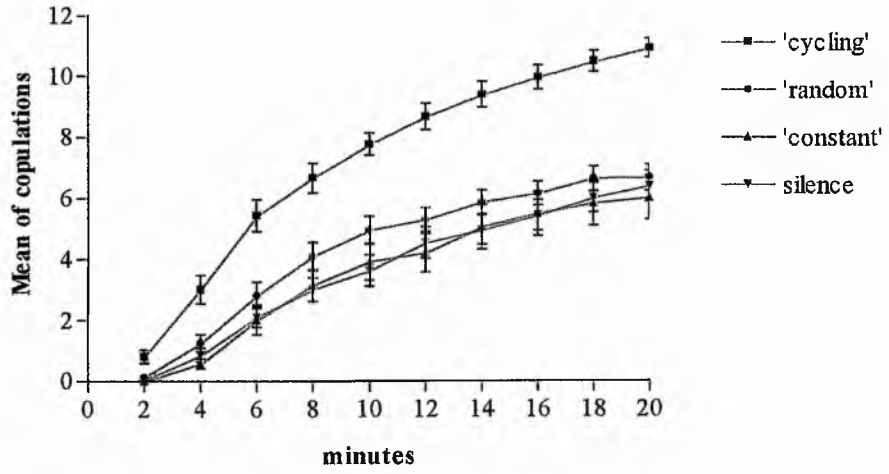
Female age, male age, time of day, block and order were tested for an influence on numbers of matings (table 2.3.2). All effects were consistent with those reported in section 1.3, except that male age was associated with differences in numbers of matings, such that older males mated more quickly (table 2.3.2). This may reflect the larger age range of males used in this experiment.

TABLE 2.3.2 *Effects of secondary factors on numbers of matings*

Factor or covariate	Test result
Block	$F_{2,839}=28.09$; $p<0.001$
Time of day	$b=-0.35$; $s=0.14$; $F_{1,839}=5.90$; $p<0.015$
Order	$F_{1,839}=0.81$; $p=0.367$
Female age	$b=0.32$; $s=0.093$; $F_{1,839}=11.69$; $p<0.001$
Male age	$b=0.28$; $s=0.095$; $F_{1,839}=8.94$; $p=0.003$

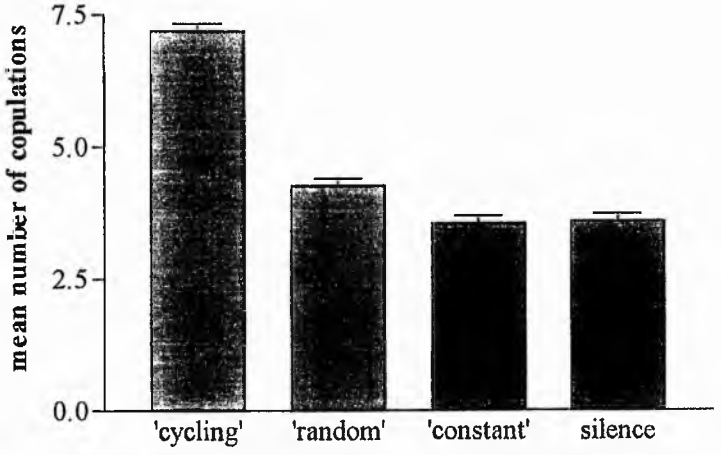
B denotes regression coefficients and s standard deviations of regression coefficients.

FIGURE 2.3.1 *Mean number of copulations with each song at each time interval*



Bars indicate standard errors

FIGURE 2.3.2 *Mean number of copulations with each song averaged over all variables*



Bars indicate standard deviations

NONPARAMETRIC ANALYSIS

The ranking of number of matings after twenty minutes was in agreement between trials when all treatments were included (table 2.3.3). The mean number of copulations after twenty minutes followed the same pattern as the rates: 'cycling' song elicited the greatest number of copulations, and there was little difference between the other three treatments (figures 2.3.1, 2.3.2 and 2.3.3).

TABLE 2.3.3 *Nonparametric analysis of number of matings after twenty minutes*

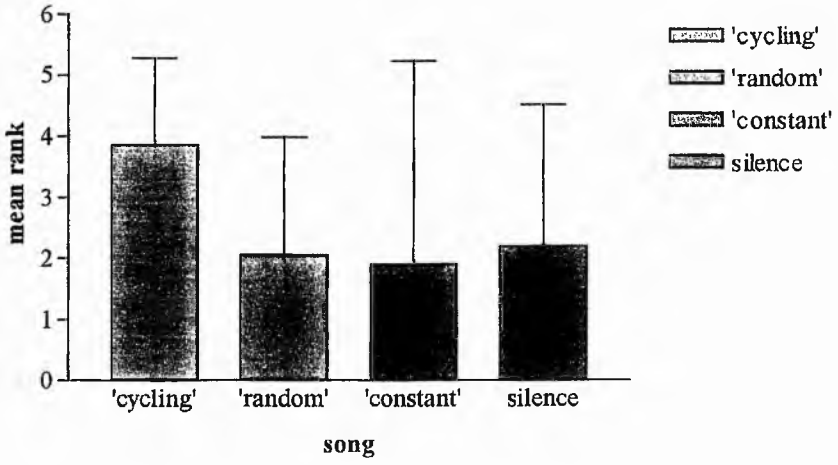
Condition	Test result
'cycling' included	W=0.52, χ^2_3 = 33.03, p<0.0001
'cycling' omitted	W=0.042, χ^2_2 = 1.76, p=0.414

W denotes Kendal's test statistic.

Comparison of the effect of 'cycling' song with the effect 'melanogaster' song of the previous chapter (sections 1.2 and 1.3) by GLM and Kendal's Test for Concordance indicated there were no differences between the two ($F_{1,409}$<0.0001, p=0.975, and W=0.142, χ^2_1 = 0.286, p=0.592). Despite considerable variability within experiments (section 1.3), this technique can be assumed to produce constant results between experiments as long as protocol and stocks do not differ, and sample size is sufficiently large.

FIGURE 2.3.3 *Mean ranks of songs*

Bars represent standard deviations.



(2.4) DISCUSSION

It has been observed that whereas cycling song increased the rate of copulation over the negative control of silence, neither song composed of randomly varying IPIs nor invariant IPI pulse song affected mating speed.

The effects of cycling and invariant songs seen here are consistent with the findings of Kyriacou and Hall (1982), who also concluded that, of the two, only cycling song stimulates female *D. melanogaster*. However, earlier publications show pulse song of constant IPI to increase female mating receptivity (e.g. Bennet-Clark and Ewing 1967, 1969; Schilcher 1976b). If it is to be assumed that the difference in the experiments is not between strains, it must be that invariant IPI stimulates females but to a much lesser degree than cycling song (slight differences were found by Kyriacou and Hall 1982), and that the present experiment was not of sufficient resolution to discern such differences. The technique adopted by Schilcher (1976b) differed from the one used in the present study in that female receptivity was measured as the mean time to the first mating occurring in a mating chamber containing five males and five females. This technique might be a more sensitive assay of sexual receptivity.

The novel element of the present study was the comparative assay of patterned and random sequences of variation in IPI. Whereas rhythmic song increased female sexual receptivity, random song did not; only the former was recognised by females as a mating signal.

This result is surprising, in view of what was noted in 2.1.1 with regard to the large amount of variation in song and typically short bursts of song within courtship. It is difficult to imagine how females detected patterned IPI oscillation here, and species-differences between periods elsewhere (Kyriacou and Hall 1982, 1984, 1986; Greenacre *et al.* 1993), particularly if the stimulus takes effect in only a short period of time, as a species-recognition signal might be expected to. That courtships may be longer than previously assumed may lend credence to the importance of IPI gradients inferred here. However, that females are apparently able to discern rates of change in IPI under the conditions used in this study cannot necessarily be taken to verify importance of song rhythms in the field. Although the artificial songs assayed here contained a similar level of variation as that of real songs, it remains to be seen whether females can distinguish between songs of different periods if they are sporadic and possibly shorter in length. The findings of this experiment do not necessarily bear relevance to behaviour occurring in the field or reflect other aspects

of the organism. The *period* gene is known to affect both the circadian rhythm and song cycle length of males (Konopka and Benzer 1971; Kyriacou and Hall 1980). 'Preference' for conspecific IPI cycle length in the laboratory (Kyriacou and Hall 1982, 1984) may be an incidental effect of some internal process resulting from a species-specific *period*-like allele having correlated effects in males and females, such that the behaviours appear coupled.

However, there is some indication that cycle length is important in mate-recognition occurring in long-term laboratory cultures. Mutation of the *period* gene modifies the length of male IPI cycle length (Kyriacou and Hall 1980; Alt *et al.* 1998) but does not alter female preference with respect to lengths of cycling song (Greenacre *et al.* 1993). The mating speed of female *D. melanogaster* in playback experiments is lower if song cycle is shortened than if songs are of conspecific or lengthened periods. However, females taken from a long term per^s culture (in which the period of IPI oscillation of males is shortened) did not differentiate between the shortened song and the other two (Greenacre *et al.* 1993). This may be a consequence of selection having acted on females to extend the range of signals which they recognised as mating signals, in order to attune preferences to the range of mating signals they were most likely to encounter. In turn, this might infer importance of IPI cycle length in determining a song's efficiency as a mate-recognition signal in laboratory cultures. If so, oscillation period is likely to constitute a significant element of signalling in the field.

It remains possible that IPI variation is the salient product of oscillation, if females require a specific amount of variation and measure it in a short space of time. The randomly varying song tested in this study contains the same amount of variation as the cycling song tested, but over the space of a whole minute. Over short time spans, say, one burst, the randomly varying song contains more variation than cycling song (see figure 2.2). Greenacre *et al.* (1993) found that female *D. melanogaster* are stimulated by cycle lengths longer but not shorter than conspecific period. It is conceivable that females of this species require some degree of variation, as they do not respond to pulse song of invariant IPI, but are not stimulated by song varying more than a specific amount over a particular space of time. The shorter IPI period of *D. simulans* song contains more variation in short spaces of time. Again, knowledge of the time taken for a courtship signal to take its effect would substantiate or refute this hypothesis.

What was previously the most likely mechanism accounting for female preference for conspecific pulse song period has been eliminated, at least in its original form.

Further investigation is necessary to resolve in more detail the mating stimulus that is constituted by rhythmic song in the laboratory, and to determine whether it plays a role in species-recognition in the natural environment. The minimum length of time required for a song to act as a signal has yet to be established, which may be a critical aid to understanding reception of song. Mating speeds of individual females can be measured (Ritchie *et al.* 1998), to see the extent in which preferences vary within populations. The appeal for more field observations made in the previous chapter is repeated. More effort can to be made in the laboratory to mimic natural conditions. In particular, males should be allowed prior sexual experience to maximise the intensity of their courtship (Scott 1986; Noor 1997). Large mating chambers should be used (Ewing and Ewing 1987) and preferences should be assayed as soon as females are sexually receptive, if behaviour is to be taken to reflect that occurring in the typically dense field populations. If possible, low levels of agitation should be attained before testing behaviour (Robertson 1982; Cobb *et al* 1987).

Perhaps the most parsimonious hypothesis as things stand is that females measure the level of IPI variation over a short space of time, and that a specific amount of variation is required to increase receptivity. This hypothesis can be tested by further playback experiments. The variation within a song over a short space of time is predicted to correlate with its influence on mating speed. Whether IPI oscillation influences female mating speed in the field as opposed to being an artefact of the laboratory can be tested by playback of songs synthesised to resemble more closely real songs. Perhaps more interruptions could be introduced and bursts made shorter, or, alternatively, real song of different species could be played to females. More investigation can be made into the difference between cumulatory and triggering signals. For example, females excitation could be raised by prestimulation with artificial sine song, and then various trigger signals could be tested. This could potentially shed light on the length of time required for a signal to take its effect.

General Summary

Playback has been shown to be a valuable method by which female preference for auditory mating signals can be measured. Artificial songs were sufficiently similar to real pulse songs for them to be perceived as mate-recognition signals. The technique was sensitive enough to detect varying strengths of preferences for different songs. Given that mating speeds were greatest for conspecific songs, the observations are likely to reflect preferences exercised in the natural environment, as opposed to being arbitrary artefacts of the laboratory. The construction and assay of a novel 'song' has permitted the properties of pulse song that stimulate females to be narrowed down. The stimulatory effects of pulse songs have been assayed independently; the observed female preferences for pulse song could not have been for other male characters correlated with pulse song.

This study provides evidence that pulse song is a critical component of the mate-recognition systems of *Drosophila melanogaster* and *D. simulans*. It was demonstrated in chapter one that female preferences for pulse song are attuned to conspecific mean IPI and/or IPI cycle length. This species-specific coupling of traits and preferences is likely to contribute towards sexual isolation of these species in the natural environment. IPI preferences appeared to be of low resolution, and the mate-recognition systems of the two species may rely primarily on different cues, both of which would be expected if intraspecific selective processes are most influential in the evolution of mating systems. In chapter two, efforts were made to localise the features of IPI periodicity which stimulate female sexual receptivity. It was indicated that the mechanism of signal detection is more complex than previously assumed. Further dissection of periodicity via playback of more novel 'songs' may help to resolve the essence of the preference.

Routes to deeper understanding of pulse song in the two contexts studied here have been outlined in each of the two chapters. Further playback experiments could dissect out the property of pulse song that stimulates females and differs between species. Measurement of female preference by playback would then be of higher resolution, facilitating measurement of individual preferences, which may be less broad than measurement of groups suggests. A preference that is more accurately scored could be subjected to genetic analysis by measurement in interspecific hybrids. Investigations of this kind have already been shown to be possible (Kyriacou and Hall 1986). Understanding the genetics underlying female preferences for pulse song

would complement investigations into the genetic bases of species differences in pulse song (e.g. Wheeler *et al.* 1991; Pugh and Ritchie 1996). The genetic basis of a trait reflects its selective history: whereas gradual change is likely to result in polygenic differences between divergent species, the spread of major mutations probably requires strong selection (Orr and Coyne 1992; McKenzie and Batterham 1994; Liu et al 1996). Although unusual genetic phenomena may be associated with speciation (Wright 1988), understanding the genetic differences between species can be extrapolated to more general mechanisms of adaptive evolution. Most genetic studies have been of signals and not preferences, yet signals should not be considered in isolation of preferences, as changes in either can potentially lead to speciation (Lande 1983; Butlin and Ritchie 1994), and species-differences in signals do not necessarily infer them to be important in speciation (Noor and Aquadro 1998).

Appendix A

Optimisation and size differences of microsatellite loci in *Drosophila sechellia* and *D. simulans*

APPENDIX A

OVERVIEW

The work carried out in chapter one of this study will contribute towards a longer-term project aiming to map quantitative trait loci (QTLs) responsible for the species difference in pulse song mean IPI between *Drosophila sechellia* and *D. simulans* (table 1.1.1.2). In essence, mean IPIs of backcross males will be correlated with their complement of marker alleles known to segregate between the two parental species. Microsatellites were chosen as marker loci because they are easily scored, and are likely to differ in size between these two closely related species as a consequence of their high variability. Optimal conditions for the amplification of three microsatellite loci in *D. sechellia* and *D. simulans* are reported. Once bands of sufficient resolution were produced for a microsatellite, the alleles were tested for size differences within and between species.

METHOD

Microsatellite loci found in *D. melanogaster* have been compiled by Schug *et al* (1998). As this species is closely related to *D. sechellia* and *D. simulans* (Caccone *et al* 1996), there is a high likelihood that the sequences flanking the repeat regions are sufficiently conserved across related species to permit amplification by the polymerase chain reaction (PCR) in the two species of interest here.

DNA EXTRACTION

The DNA of single flies was prepared as follows:

- 'Squishing buffer' was prepared by mixing 500μL (10mM TrisHCl (pH 8.2), 1mM EDTA, 25mM NaCl) with 5μL pronase (200μg/mL).
- Each individual fly was placed in an Eppendorf tube and crushed using a yellow tip containing 60μL of squishing buffer, which was then expelled into the tube.
- Each mixture was incubated at 37°C for 30 minutes, following which heating to 95°C for 2 minutes inactivated the enzyme.

DNA preparations were stored at –20°C.

AMPLIFICATION

PCR amplification was carried out in 10μL reaction mixtures each containing 1μL DNA template, 0.3μM of each primer, 0.32mM dNTP, 1.5-3.0 mM MgCl2 (table A1), 10μL *Promega* 10x buffer and 1 unit of Bioline *Taq* polymerase. Samples were overlaid with mineral oil. 40 cycles were run on a thermal cycler under the following conditions: 10 seconds at 92°C (denaturation), 15 seconds at 45-57°C (table A1) (annealing) and 1 minute at 72°C (elongation). In addition, an initial denaturation step of 92°C for 2 minutes and a final elongation step of 72°C for 5 minutes were included in the programme. PCR products were visualised on 2% TBE agarose gels stained with ethidium bromide. Table A1 gives the optimal concentration of magnesium chloride and annealing temperature for the three loci.

TABLE A1 *Optimal conditions for microsatellite amplification*

Name of gene (in *D. melanogaster*)	**Primer sequence (5' to 3')** forward, reverse	**PCR conditions** mM $MgCl_2$, °C
grainyhead	Tccacgacaacgatctcgca, Ctaacaatgtcgccgggatg	1.5, 57
ovo	Aatgggtacatcaactgtgg, Gagtattatgccgtaggctg	3.0, 45
slowpoke	Agtggcaataagtatggccg, Cgacgtcttgaacgatctca	2.0, 52

SIZE DIFFERENCES OF ALLELES WITHIN AND BETWEEN SPECIES

Once clear bands had been resolved for both *D. sechellia* and *D. simulans*, PCR products from a number of individuals of both species (8 *D. sechellia* and 4 *D. simulans* individuals) were run out and visualised as above. A 1-kilobase DNA 'ladder' (*Gibco*) was run alongside the PCR products. No size differences between individuals of the same species were apparent, except *α–spec*, for which one *D. sechellia* was approximately 10 base pairs longer than the others. Whether or not the alleles from the two species differed in size is given in table A2 (which contains additional loci optimised by the same means as described above). Alleles that were species-specific were used as marker loci.

TABLE A2 *Species differences in microsatellite alleles*

Name of gene (in *D. melanogaster*)	**Size difference between species?**
α–spec	No
Cactus	No
caudal	Yes
Csp	No
Drosophila G-protein α–subunit	Yes
deltex	Yes
grainyhead	Yes
Heat shock protein 67	No
ninaC	No
pros	No
scute	Yes
sex lethal	No
twist	No

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